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THE CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF IMMUNO-REGULATORY CELLS IN MURINE BONE MARROW

by

James Dean Michelson

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With thanks to three immunologists:

Michael Iverson - who endeavored to teach me the foundations of immunology;

Richard Gershon - who was my guiding beacon to the future of immunology;

and Mark Horowitz - a friend, who showed me how to bridge the gap between theory and experiment.

This thesis is dedicated to my parents:

my father, who has set an example to which I will always aspire,

and my mother, whose memory is forever with me.

SUMMARY

Recent years have witnessed a growing appreciation for the complexity and wide range of functions performed by lymphocytes. This has been particularly true for bone marrow cells (BMC) which have been noted to possess cells involved in hematopoiesis, lymphopoiesis, and bone metabolism. It has been repeatedly demonstrated that the bone marrow (BM) contains a potent suppressor cell of uncertain phenotypic identity, the physiologic significance of which has been only speculated upon without the benefit of decisive data.

This thesis describes the discovery of a cell in the BM that reduces the suppressive activity of the bone marrow suppressor cell (BMSC), and outlines an integrated BM physiology based upon the interactions of the BMSC and its modifier cell, the contrasuppressor. Data is presented that shows the BMSC to be lacking all standard lymphocyte or macrophage cell surface markers while its suppressive activity differs from that of the classical T suppressor cell by its extraordinary range of action, its lack of a secreted soluble suppressor factor, and its failure to require Ly-2,3^{+} cells for suppressive activity. The contrasuppressor, which is preferentially expressed in neonatal mice and certain CBA substrains, is a Thy-1^{+} cell that is also extremely sensitive to a factor, probably an immunoglobulin, present in normal rabbit serum.

The possible physiologic roles of these cells is demonstrated in experiments involving the splenic colony forming units (CFU-S) assay as a model of hematopoiesis and in experiments in which BMC were exposed to parathyroid hormone (PTH) as a stimulant of osteo-

clastic activity. The data presented shows that maneuvers leading to reduced contrasuppression resulted in lower hematopoietic activity, as measured by the CFU-S assay, and, conversely, that the BM of anemic mice who had high endogenous levels of hematopoiesis also demonstrated elevated BM contrasuppressor activity. It was also found that BM suppression was markedly relieved by exposure to PTH, indicating that at least one PTH-specific cell was present in the contrasuppressor circuit.

The work of others concerning lymphocytic involvement in experimental models or disease states in both hematopoiesis and bone metabolism is discussed in light of the data presented. Observations are made concerning the concept of contrasuppression as it may operate in a general sense as well as its use in the unique microenvironment of the bone marrow. The bone marrow contrasuppressor is compared to contrasuppressors described in other systems and its significance in an integrated BM physiology is speculated upon.

INTRODUCTION

It is said that the medieval physician Galen "considered the thymus to be nothing more than a pad protecting the heart from the boney sternum" (1). Subsequent advances in the understanding of thymic production of cellular blood products were scarce, not withstanding Metchinkoff's investigations in cellular immunity in the late 1800's (2) and a widely recognized association between thymic abnormalities and hypoplasia of one or more hematopoietic cell lines (1). It was not until 1966 that the immunologic function of lymphocytes was clearly shown by Gowans (3). Building upon the work of Miller, who showed in 1961 that neonatal thymectomy resulted in numerous immunological defects (4), Raff, in 1969, demonstrated the thymic dependence of lymphocytes bearing the θ surface antigen (5).

It had been previously observed by Ovary and Benacerraf that the use of a conjugated hapten-carrier immunogen restricted the secondary immunologic response characteristics in a way that suggested carrier-specific regulation of the hapten immunogenicity (the so-called carrier effect) (6). When Claman, et al (7), demonstrated immunological synergism between thymocytes and bone marrow cells, it was quickly appreciated that a 2-cell model of immunocompetence would best explain these phenomena (8). Confirmation was presented by Raff, who demonstrated that a carrier-specific θ^+ lymphocyte, which he called a helper cell, cooperated with a hapten-specific θ^- lymphocyte to generate the carrier effect (9).

The discovery of suppressor cells by Gershon in 1971 (10) was the first hint of true homeostatic immunoregulation at the cellular level. With the delineation of an uncommitted θ^+, Ly-1,2,3$^+$ pool of lymphocytes that could generate either help or suppression depending upon the intermediate homeostatic requirements (11), it was not long before a fully integrated feedback suppression circuit was elucidated (12). Even before the glue had set on the suppressor circuit model, it was hypothesized that yet another level of control, involving the suppressor cell, was acting in symmetry with the regulation of helper cells. This concept, dubbed contrasuppression by Gershon in 1974 (13), was an abstraction until 1980, when it was first demonstrated experimentally (14,15,16). Briefly, it can be viewed as the inverse of feedback suppression, in which a high level of helper cell activity induces the creation of suppressor cells via a pool of uncommitted precursor lymphocytes. Analogously, in the contrasuppressor circuit, a high level of suppressor cell activity serves to induce, via the precursor pool, contrasuppressor cells which subsequently act upon helper cells to render them resistant to suppression (16). Further discussion of this superficially confusing circuit can be found in Appendix I.

Paralleling the increasingly sophisticated modeling of immune regulation was a growing awareness that lymphocytes could participate in non-immunological roles. Of particular interest has been the cellular milieu of the bone marrow (BM) as it anatomically provides close contact between cells of the lymphocytic and hematopoietic lines as well as those concerned with bone metabolism. While the ultrastructure of the BM influences differentiation among its resident cellular populations (17), it is also true that specific cell-to-cell interactions can alter bone marrow cell functions.

Several investigators have demonstrated a suppressor cell in the BM that has been termed a large lymphocyte (18), possibly a B cell (19,20,21) or a pre-T cell (22) which, although potently suppressive in an *in vitro* lymphocyte culture, also was capable of non-specifically stimulating DNA synthesis in spleen cells (23). With the exception of its suppressive nature, however, none of these characterizations of the BM suppressor cell (BMSC) have been consistently demonstrated (20,24, 25).

If the BMSC is, in fact, a lymphocyte, then the question of its function can be raised in the context of its predominantly non-immunologic surroundings. The obvious issue of BMSC regulation of BM antibody production has been addressed by others (23,26) and will be discussed following the presentation of pertinent data. Pursuant to the larger concept of non-immunological roles for lymphocytes, data in the literature suggests that T cells, or their products, may be an important factor in the regulation of hematopoiesis and/or bone metabolism. Several experimental models of hematopoiesis have demonstrated some form of thymic (27,28), thymocyte (29,30,31), or T lymphocyte dependence (32,33). In addition, many disease states involving altered bone metabolism have been associated with T cells (34) or T cell products (35,36). Many of the experiments presented here were designed to detect just such interactions among the physiologic systems under examination.

It is easily appreciated that for a dynamic system to be regulated, it must possess opposing regulatory mechanisms that prevent degeneration to either a state of uncontrolled activity or one of total shutdown. In the BM, where a seemingly omnipresent, non-reactive suppressor cell resides, it might be expected that an opposing cell, a contrasuppressor,

would be found that provides the BM with the flexibility to respond to changes in its microenvironment. Such a cell was, in fact, discovered. The implications of this finding will be discussed in the context of an integrated BM physiology.

METHODS

I. Mice

A. Adult strains: All adult mice were 6-10 weeks old and had been housed in the Yale mouse colony for at least 1 week after arrival from outside suppliers. The following strains were obtained from Jackson Labs: AKR, A.TH, Balb/ByJ, Balb/ByJ nude, Balb/c, C57BL6, CB.20, CBA/CaJ, CBA/H, CBA/J, DBA/2, NZB and SJL. C57BL6 were also obtained from Charles River Labs on a few occassions. The following mice were bred and generously donated by D. Murphy: $(B6xA)F_1$, $(B6xD2)F_1$, $(B6/TL^+xA)F_1$, B10.A(1R), B10.A(3R), B10.A(4R), B10.S, $C_2D_3F_1$ and M16. J. Kemp kindly provided homozygous CBA/N females and $(C57BL/6 \times CBA/N)F_1$ males (defective F_1's) and females (non-defective F_1's) (37). C. Janeway generously provided Balb/c mice that had been injected from their first feeding with anti-mouse immunoglobulin (Ig) (50µl of concentrated solution). The mice were injected similarly 3 times per week thereafter until their sacrifice at 8 weeks of age, while control mice were injected with normal rabbit serum according to the same protocol. At sacrifice, the experimental mice were shown to have no cells with detectable surface Ig (38). C. Janeway also provided $B6AF_1$ "B" mice that had been thymectomized, lethally irradiated, and reconstituted with anti-θ + complement treated syngeneic bone marrow.

B. Neonatal strains: Pregnant female mice were obtained from Jackson Labs, with their offspring being used for experiments at 7 to 13 days of age. Strains used were: C57BL6, A.TH, Balb/ByJ, and CBA/J. D. Murphy donated similarly aged neonates of the following strains: B10.A(1R), B10.A(3R) and B10.S.

C. Bleeding: In one set of experiments, CBA/J mice were made anemic by alternate-day bleeding with an average blood loss of 0.3ml, for at least 2 weeks. The hematocrit at sacrifice ranged between 30-35%. Normal mouse hematocrit is roughly 55%.

D. Sacrifice: All adult mice were sacrificed by cervical dislocation while for neonatal mice, decapitation was required.

II. Cell Suspensions

A. Spleen cells (WS): Spleens were removed from the mice using sterile technique, then gently ground between the frosted ends of two sterile microscope slides to form a single-cell suspension in phosphate buffered saline (PBS) containing 2% fetal calf serum (FCS). After the cells were washed three times and all debris removed, the cells were diluted to a concentration estimated to be between 10^7 and $2x10^7$ cells/ml and an aliquot counted by Trypan blue exclusion.

B. Bone Marrow Cells (BMC): After disarticulation of the rear limbs, all overlying soft tissue was stripped from the femurs and tibias by sharp dissection. Using sterile technique, the metaphyses on either ends of the bones were removed and the marrow cavity was vigorously flushed with PBS with 2% FCS using a 27 gauge needle. After three washings, the cells were diluted and counted as for the WS. All nucleated BMC were included in the cell count for the BM.

III. Cell Selection Techniques

A. Antisera: Antisera treatments consisted of incubation of the cells in the antisera (diluted in Balanced Salt Solution (BSS) with 5% FCS according to cytotoxicity data generated within the Gershon

lab) for 30 minutes at $4^{o}C$ followed by a wash and incubation of the cells in complement (diluted 1:5 in BSS) for 30 minutes at $37^{o}C$. After at least 2 washings in PBS with 2% FCS, the cells were counted by Trypan blue exclusion. Antisera used were: anti-Thy-1.2 (rat hybridoma monoclonal antibody, a gift of J. Sprent), rabbit anti-mouse brain (Litton Bionetics, Kensington, MD), anti-K^{s} and anti-I-J^{b} (both gifts of D. Murphy). F.W. Shen very generously donated the following heterologous antisera: anti-Ly-1.2, anti-Ly-2.2, anti-Qa-1, anti-Qa-2/3, anti-N.K, and anti-Ly-5.1. Rabbit complement (RC) was either low tox (Cedar lane Labs) or pooled complement screened in Gershon's lab for low background cytotoxicity towards mouse spleen cells. Pooled guinea pig complement (GPC) was similarly screened before use.

B. Positive Selection Methods:

1. Ig plates - Plastic petri plates coated with purified goat anti-mouse Ig were used to selectively retain B cells, which possess surface Ig (sIg), after the method of Wysocki and Sato (39). Briefly, after 70 minutes of incubation at $4^{o}C$, mouse B cells selectively adhere to the coated plates while the T cells that elute off with gentle pipeting are less than 5% contaminated with B cells. Further incubation of the plates at $37^{o}C$ for 30 minutes allows the recovery of the B cells, which are less than 1% contaminated by T cells by fluorescence.

2. Fc plates - Selection of Fc receptor positive (FcR^{+}) cells was accomplished by a modification of the method of Parish (40). Briefly, sheep red blood cells (SRBC's) coated with mouse anti-SRBC antisera were used to cover the bottom of plastic petri dishes that

had been coated with poly-L-lysine. Cells were incubated on these plates for 45 minutes at 4°C, with non-adherent cells then removed by gentle pipeting. Adherent cells were removed by osmotic shock and vigorous pipeting.

3. Vicia Villosa (V. Villosa) - The lectin V. Villosa was placed on plastic petri plates and used for cell selection as described by Green (16). Briefly, cells were incubated on the plates for 30 minutes at 37°C, at which time non-adherent cells were removed by gentle pipeting. Addition of n-acetyl-galactose amine (the specific sugar for the lectin) and incubation for 10 minutes at room temperature allowed the elution of the adherent cells. Previous studies have shown that macrophages do not elute from the plates, Ly-2^+ cells are non-adherent, T helper cells are not adherent and roughly 50% of T contrasuppressor cells are in the adherent population (41).

C. Complement Fractionation: Pooled rabbit complement (RC) screened for low cytotoxicity against mouse WS was used to obtain a solution enriched for rabbit Ig (RIg) after the method of Mishell and Shiigi (42). Briefly, rabbit serum was added to an equal volume of a saturated ammonium sulfate solution (defined at 4°C). The precipitate formed was centrifuged at 10,000 rpm for 10 minutes and resuspended in PBS. After repeating this sequence twice more, the resulting solution was dialyzed against PBS for 24 hours with one change of dialysate. The final solution was used at either a 1:10 or 1:20 dilution as an antiserum.

D. Complement Absorption: To absorb organ-specific antibodies from RC, equal volumes of the serum and either murine BMC or WS were

mixed together and incubated for 30 minutes at $4^{o}C$. After removal of the cells, the absorbed serum was used as a source of complement at a 1:3 dilution if retained complement activity was satisfactory in a standard cytotoxicity assay.

F. Use of Cells After Fractionation: With all the preceding selection techniques, the cells of the resulting subpopulations were added to, for instance, an in vitro culture in equivalent measure to their proportion of the unseparated cell population. For example, if the sIg^{+} BMC constituted 1/3 of the starting cell population, then $1x10^{6}$ sIg^{+} BMC would be added where $3x10^{6}$ unseparated BMC would be used. In antisera treatment, the complement control was used as the equivalence standard. This was done to control for the effects of RC upon BMC. Doing so removed a large source of artifact, although it probably reduced the sensitivity of the experiments involving the contrasuppressor cell to some extent.

IV. Treatment of BMC

A. Thymopoietin (TP): TP (a gift of G. Goldstein) is a polypeptide extract of thymus that was known to induce cell surface θ on some lymphocytes (43,44). It was dissolved in RPMI 1640 with 2% FCS at concentrations between 1 and 10^{-3} μg/ml. $1x10^{7}$ BMC/ml were incubated in the TP solution for 2 hours at $37^{o}C$, washed and counted by Trypan blue exclusion.

B. Parathyroid Hormone (PTH): Powdered salmon PTH (a gift of R. Baron) was dissolved in RPMI 1640 with 2% FCS to a concentration of 1ug/ml (0.1μM). $1x10^{7}$ BMC/ml was incubated in the PTH solution for 3.5 hours at $37^{o}C$, washed and counted by Trypan blue exclusion. Control BMC were similarly incubated in RPMI 1640 with 2% FCS.

V. Cell Cultures

A. Cultures: WS and BMC were cultured *in vitro* according to a modification of the Mishell-Dutton method (42). Briefly, WS ($5\text{-}10\text{x}10^6$/ml) and SRBC's (25λ of 1% SRBC) were suspended in roughly 1ml of culture media containing RPMI 1640, FCS, antibiotics, a fungicide, and 2-mercaptoethanol. Individual cultures included WS plus SRBC, to which BMC suspended at a concentration of $30\text{x}10^6$ cells/ml could be added. All experimental groups were represented by triplicate culture wells in Falcon 3008 culture plates. Primed WS was obtained from mice that had been injected with 0.2ml of 1% SRBC, intraperitoneal, 5 days before sacrifice. Cells were cultured for 5 days at 37°C in an incubator containing a 5% CO_2 atmosphere.

B. Assay: Cultures were assayed via the Cunningham modification of the Jerne method (45). Briefly, after removal of cells from culture, they were washed and brought up to 1ml in BSS. Of this, 100λ was added to 25λ of 10% SRBC, 50λ of BSS, and 25λ of GPC (diluted 1:3 in BSS) and placed in a Cunningham slide chamber, the sides of which were then sealed with wax. After incubation for 1 to 1.5 hours at 37°C, the slides were examined for clear plaques, each of which represented an IgM anti-SRBC secreting cell. The number of plaques in each chamber was multiplied by 40 (to account for cell dilution in the assay) to yield the total plaque forming cells/culture (PFC/culture). Each well was individually assayed and triplicates were averaged to obtain the PFC/culture for each experimental group. Suppression within experiments was determined by comparison to control WS cultures and was calculated by the formula:

$$\% \text{ suppression} = \left(1 - \frac{\text{experimental PFC/culture}}{\text{control PFC/culture}}\right) \times 100\%$$

C. Conditioned Culture Media: In one set of experiments, media obtained from one to five day old WS + BMC + SRBC cultures was mixed with an equal volume of fresh media to make conditioned culture media.

VI. Colony Forming Units Assay

The generation and measurement of colony forming units in the spleen (CFU-S) was carried out as described by Till and McCulloch (46). In brief, mice were exposed to whole-body x-irradiation totaling 900 rads or 500 rads and were then injected with 0.2ml of syngeneic donor mouse BMC suspension by lateral tail vein between 2 and 5 hours after irradiation. Although 500 rads of whole-body irradiation is not lethal, as 900 rads is, the fraction of endogenous CFU-S that survive is less than 1% (47), so that the data from these mice can be reasonably compared to the lethally irradiated mice. This is supported by the lack of any differences seen in those experiments in which both dosages were used. The recipient mice were housed in laminar flow hoods, with tetracycline in their water, for 7 to 10 days, at which time they were sacrificed and their spleens removed. After fixation in Bouin's solution for 18-24 hours, the spleens were examined under 1.7 x magnification for white nodules, each of which represented a CFU-S arising from one cell (48). Control irradiated mice in which no cells were injected never yielded more than 1 CFU/spleen and usually were devoid of any CFU-S.

VII. Statistics

All statistics were derived from a 2-tailed student's t-test for either paired or unpaired data, as indicated.

RESULTS

BM Suppression

The phenomenon of BM suppression is shown in Table 1. In these experiments, the response of WS to SRBC's in *in vitro* culture conditions is modified by the addition of syngeneic BMC to the culture. Table 1 demonstrates how the addition of as few as $1x10^6$ BMC to $5x10^6$ WS frequently suppressed the WS response to less than 50% of control WS values for both primary and secondary WS responses. In all cases $3x10^6$ BMC was profoundly suppressive. Figure 1 illustrates the roughly linear dose response curve that results as the addition of increasing numbers of BMC progressively decreases the PFC response.

That this phenomenon is not particular to C57BL6 mice is apparent from Table 2, which shows that of 14 mouse strains tested, all significantly suppressed a syngeneic WS response, 13 to less than 50% of control cultures, while one completely abolished the generation of PFC's.

Phenotype of the BMSC

Identification of cell-surface markers on the BMSC was carried out using both positive and negative selection techniques, as shown in Tables 3 through 7. In Table 3, experiments 1-4, utilizing antisera directed at several standard T cell markers, were unsuccessful in removing BM suppression. Experiment 5 also indicates that cells with phenotypes known to characterize natural killer cells (49) do not appear to mediate the BM suppression. Experiments 6 and 7 demonstrate, however, that the BMSC does carry surface major histocompatibility antigens, since anti-H-2 antisera + complement (C) treatment of BM removes its suppressive activity. The non-T cell nature of the BMSC is confirmed in Table 4, in which it can be seen that mice lacking T cells, namely athymic nude mice and "B" mice, are able to generate potent BM suppression.

Induction of surface Thy-1 antigen was attempted, Table 5, by preincubation of BMC in thymopoietin solutions. If the BMSC is a precursor T cell, this maneuver should either deplete the ranks of BMSC's through maturation, or make the BMSC a Thy-1^+ cell. It is clear from these experiments that neither situation exists, since both control and anti-Thy-1.2 + C treated BMC's are profoundly suppressive.

The possibility of surface Ig (sIg) expression by the BMSC is addressed by two different methods in Table 6. The first 2 experiments, utilizing goat anti-mouse Ig coat petri plates to positively select for sIg^+ cells, demonstrate that following BM fractionation, the suppressive activity migrates with sIg^- fraction. Experiments in which the BMC were first preincubated with dibutyryl-CAMP to induce sIg prior to sIg fractionation yielded identical results (data not shown). Mice who have had chronic exposure to an anti-μ antiserum from birth have been shown to possess little or no sIg^+ cells (38). It is easily appreciated from experiments 3 and 4 that such mice are not depleted of their BMSC's.

Finally, in Table 7, the BMSC was tested for the presence of an Fc receptor (FcR), as determined by cell adherence to SRBC's coated with goat anti-SRBC antiserum. This experiment shows the adherent fraction (i.e. - the FcR^+ cells) possesses very little suppressive activity while the non-adherent, FcR^-, fraction is quite suppressive.

Mechanism of Action of the BMSC

Although the BMSC was not an Ly-$2,3^+$ cell, there was the possibility that it was recruiting Ly-$2,3^+$ cells in the WS to generate suppression. This possibility was ruled out by experiments, depicted in Table 8, in which the removal of Ly-$2,3^+$ from the WS did not alter the degree of

suppression delivered by BMC's. Experiment 1 extends this result by showing that the complete removal of Ly-2,3$^+$ from WS and BMC's was also without effect, as indicated by the 98% suppression of the WS response seen under these conditions.

In Table 9, supernatant media from the usual BMC + WS + SRBC cultures was obtained from one to five day old cultures and subsequently used as conditioned culture media to explore the possibility of a soluble factor that would mimic the BM suppression. When such media was used on WS + SRBC cultures, absolutely no suppressive effects were observed, indicating that the suppression generated by BMC's is not mediated by a stable soluble factor.

Genetic Restriction of BMSC Activity

Given the ubiquitous presence of the BMSC in the mouse strains tested, an analysis of the genetic restrictions of its activity was undertaken, with representative results shown in Table 10 and 11. Experiments 1 through 3 in Table 10 demonstrate that limited, well-defined genetic mismatching between the BMC and WS does not alter the BM suppression observed. While these experiments show that Mls, U kappa and V_H mismatches are irrelevant, experiment 4 furthers this observation to include the background genome. Here suppression was seen even when the BMC-WS homology was limited to the H-2 and Mls loci.

In Table 11, it can be seen that genetic mismatching between the BMC and WS, to the extent of being essentially random, has no effect upon the BM suppressive activity. Experiments 1 through 3 show that H-2 mismatching, in the presence of assorted other genetic differences (including Mls, U kappa, and V_H) still resulted in BM suppression of the WS response.

BM Suppression is Less in Neonatal Mice

BMC of neonatal (less than 14 days old) mice, was cultured *in vitro* with syngeneic WS to examine its suppressive activity. Table 12 gives representative examples of many such experiments. While experiments 1 and 3 used unprimed WS and experiment 2 used SRBC-primed WS, all three showed that neonatal BM much less suppressive than its adult counterpart. Even in the least dramatic example, experiment 3, the neonatal BM generated only half the suppression expressed by the adult BM. In general, the addition of neonatal BMC's to WS + SRBC cultures resulted in less than 30% suppression of the WS response (pooled data from more than 15 separate experiments). Figure 2 shows two other experiments in which the dose-dependent BM suppression of neonatal BM is compared to adult controls. At every dose tested, neonatal BMC's were clearly less suppressive than its adult control.

Table 13 shows 3 representative examples of efforts to characterize this phenomenon. In these experiments, as in all similar ones performed, treatment of the neonatal BMC with anti-Thy-1.2 + C resulted in increased suppression. Of note is that the complement controls also reflect a moderate increase in suppression. This effect, which will be discussed later, was most pronounced with rabbit complement and prevented the use of anti-Ly antisera (all of which are compatible only with rabbit complement) for further phenotyping. The experiments in Table 13 used guinea pig complement.

While these experiments indicate that a Thy-1^+ cell was reducing neonatal BM suppression, the relevance of this phenomenon to BM suppression in the adult was not *a priori* straight forward. Through simple mixing experiments, presented in Table 14, it was possible to determine

what effects, if any, the neonatal BMC's might have upon the adult BMSC. Experiments I and II show that the addition of $1x10^6$ neonatal BMC to $3x10^6$ adult BMC reduces the adult BM suppression by up to 21%. Furthermore, as the proportional contribution of neonatal BMC towards the total number of BMC used increases, the level of suppression decreases. This demonstrates that the neonatal BM contains a contrasuppressor activity that reduces the suppressive activity of the adult BMSC.

Increased Contrasuppressor Activity is Seen in Some Adult Mice

In the process of determining the strain distribution of the BMSC, it was discovered that 3 CBA substrains showed reduced levels of BM suppression as adults. Table 15 shows representative examples demonstrating that the CBA/CaJ, CBA/H, and CBA/N mice have markedly less suppressive BM than CBA/J mice. From Table 16 it can be further said that this low level of BM suppression can be largely, if not entirely, ascribed to a Thy-1^+ cell that masks BMSC activity. This is in agreement with the results obtained for neonatal BM, making it likely that the CBA/CaJ, CBA/H, and CBA/N mice possess a contrasuppressor cell similar, if not identical, to the neonatal contrasuppressor.

The Contrasuppressor is Not Simply a Helper Cell

To answer the question of whether the contrasuppressor was simply a helper cell that did not directly affect the BMSC activity, BMC's adherent to the lectin V. Villosa, which does not bind T helper cells (see Methods), were used as a source of contrasuppressor cells. Table 17 shows two experiments which demonstrated that the V. villosa adherent cell population was depleted of BM suppression. Experiment 2 also shows that the lack of adherent cell suppression is due to an enriched contra-

suppressor population since anti-Thy-1 + C treatment of these cells results in profound suppression. Equally clear is that the adherent cell population is unable to deliver help to B cells, making it extremely unlikely that the BM contrasuppressor is simply a helper cell.

Rabbit Serum Contains an Anti-Mouse BM Contrasuppressor Activity

It had been noted in many experiments that treatment of BMC with rabbit complement (RC) served to increase suppression. Although this was most noticeable in those mice with high levels of contrasuppression, it was detectable in virtually all mice. Because this severely limited the ability to phenotype the contrasuppressor cell, a closer examination of this phenomenon was undertaken.

As shown in Table 18, initial experiments demonstrated that heat-inactivated RC possessed decreased anti-contrasuppressor activity than untreated RC, as reflected by the greater suppression manifest by RC-treated BMC than by heat-inactivated RC-treated BMC. While this did not identify the anti-contrasuppressor cell activity beyond implicating the complement cascade as a necessary component, experiment 2 implied that an antigen-specific factor was involved. In this experiment, RC absorbed with BMC was relatively ineffective for removing the contrasuppressor, although it still contained an intact complement pathway (see Methods). The specificity of the RC factor can be inferred from the negligible effect of absorbing the RC with spleen cells.

Table 19 shows experiments that further identify the RC antigen-specific factor. In Experiments 1 and 2, heat-inactivated RC was used as a source of RC factor and guinea pig complement (GPC) - which has low anti-contrasuppressor activity - was used as a complement source. In both cases, the cytotoxicity of GPC was virtually doubled by the

addition of heat-inactivated RC. A more clear-cut example is Experiment 3, in which the ammonium sulfate precipitable fraction of RC - a fraction that is at least 70% Ig (see Methods) - is used as an RC factor source. This experiment clearly shows that the cytotoxic factor in RC is contained in the ammonium sulfate precipitable fraction and is likely of immunoglobulin origin.

Regulation of BMC-Derived CFU-S and Hematopoiesis

In an attempt to discover the physiologic significance of the BM regulatory circuit elucidated from in vitro cultures, the effect of perturbating the BMSC-contrasuppressor cell balance upon BMC-derived colony forming units in spleens (CFU-S) was investigated. Approaching this question through standard techniques, BMC were Thy-1 depleted by anti-Thy-1 + GPC treatment, using GPC that was screened for low anti-contrasuppressor cell activity. Figure 3 gives the results of 3 experiments in which untreated or Thy-1 depleted BMC were adoptively transferred to irradiated syngeneic mice. Using CBA/N (Experiment 1) or CBA/CaJ (Experiments 2 and 3) mice to maximize the sensitivity of the experiments, it was found that Thy-1 depleted BMC expressed fewer CFU-S in all experiments.

Figure 4 presents the results of other pilot experiments, in which untreated or RC-treated BMC were adoptively transferred to irradiated syngeneic mice. It can be seen that, in every case, RC treatment of BMC reduces CFU-S expression. The possibility of a RC-sensitive CFU-S "helper" cell is addressed in Table 20, in which untreated and RC-treated BMC are given alone or in combination to irradiated mice. In both experiments the RC-treated BMC not only express fewer CFU-S than

untreated BMC but, when the former is added to the latter suppression of untreated BMC CFU-S expression is apparent. This implies that a suppressor cell for CFU-S expression is being unmasked by RC-treatment of BMC.

In apparent conflict with these results were the experiments in Table 21, in which adult and neonate BMC CFU-S generation are compared. These two experiments clearly show that the adoptive transfer of neonatal BMC resulted in significantly fewer CFU-S than similar numbers of adult BMC. This is the opposite of what would be expected if CFU-S expression was primarily determined by the level of contrasuppression.

Addressing the question of the physiological role of BM contrasuppression from another direction, mice were made anemic through periodic phlebotomy and their BMC was tested for in vitro suppression. Figure 5, which shows the results of two such experiments, demonstrates that anemic BMC produced only half the level of suppression manifested by the control BMC. Furthermore, after treatment of the anemic BMC by anti-Thy-1 + GPC, the BMC suppression increased from 18% to 31% in Experiment 1 and from 21% to 47% in Experiment 2. Regardless of whether the anti-contrasuppressor agent is the antisera or the GPC, it is clear that increased contrasuppression is contributing to the lower BM suppression seen in anemic mice. In addition, simple dilution of the BMSC secondary to active hemoproliferation is an unlikely explanation for the reduced BM suppression as the anemic BM was not any more cellular than the control BM.

The Effect of Parathyroid Hormone on BM Suppression

In further explorations of BM physiology, the effect of parathyroid hormone (PTH), which increases osteoclast activity in bone, upon *in vitro* BMC cultures was examined. Figure 6 shows the results of 3 such experiments in which the pretreatment of BMC with PTH - note that the PTH is *not* present during *in vitro* culture - significantly ($P < .05$) decreases the BM suppression generated by those BMC. A dose-response curve of BM suppression delivered by PTH-treated BMC, Figure 7, indicates that the PTH treatment alters the kinetics of BM suppression, as reflected by the different slopes seen for control and PTH-treated BMC.

In summary, the data presented depicts the interactions ot two cells found in murine bone marrow. The first, partially described by others (19,20,21,22,24,25), is the BMSC, which can be seen to be a cell lacking in all standard lymphocyte markers (Tables 3,4 and 6). This cell is further differentiated from the classical T suppressor cell by the former's ability to suppress allogeneic lymphocyte responses (Table 10 and 11).

The second, a newly described cell called a BM contrasuppressor, acts to reduce the suppression generated by the BMSC (Table 14). This cell is Thy-1^+ (Table 13) and is found in certain adult CBA mice (Table 16), but is definitely not a T helper cell as it does not aid B cells in the production of antibodies (Table 17). Furthermore, the presence of BM contrasuppression correlates with increased hematopoietic activity, either as measured in the CFU-S assay (Figures 3 and 4, Table 20) or as seen as an accompaniment to *in vivo* anemia (Figure 5). Finally, PTH treatment of BM results in reduced *in vitro* BM suppression (Figures 6 and 7), implying that the balance between BM suppression and contrasuppression may be altered by activation of a PTH receptor.

TABLE 1

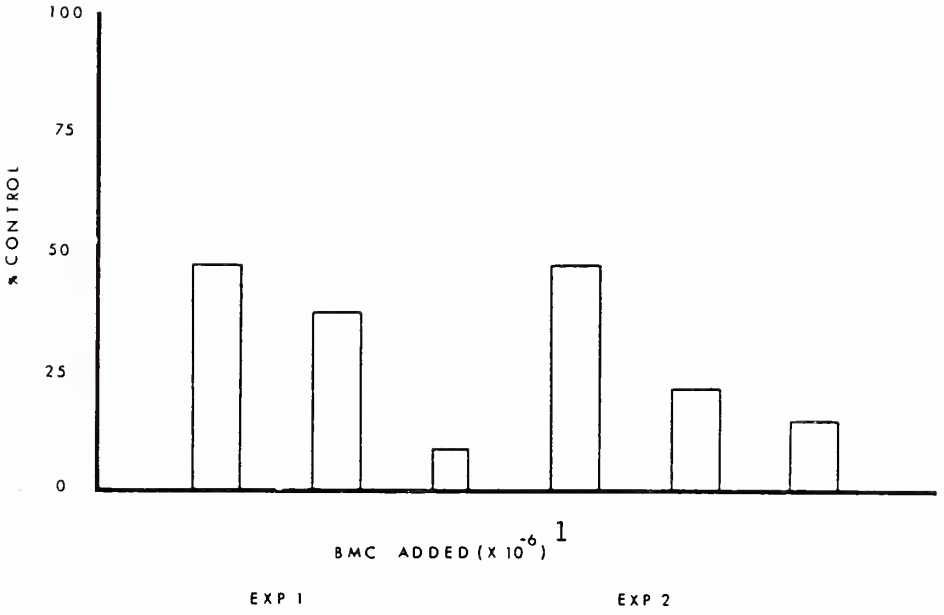
BMC SUPPRESS BOTH PRIMARY AND SECONDARY IN VITRO SPLENIC ANTI- SRBC RESPONSE

Experiment	BMC Added	PFC/Culture[1] Primary	PFC/Culture[1] Secondary	% Suppression
I	None	2387		—
	1×10^6	893		63
	3×10^6	13		100
	None		4427	—
	1×10^6		2560	42
	3×10^6		80	98
	3×10^6 BMC cult. alone	13		—
II	None	1520		—
	2×10^6	1093		28
	3×10^6	200		81
	None		8000	—
	2×10^6		427	95
	3×10^6		293	96

1. BMC mixed with 5×10^6 WS for in vitro culture (see Methods). Spleen cells were from naive mice ("primary") or from mice innoculated at least 4 days before sacrifice with 0.2 ml of 1% SRBC i.p.("secondary"). All cells obtained from C57B16 mice.

FIGURE 1

BMC SUPPRESSOR ACTIVITY IS DOSE DEPENDENT



1. C57B16 BMC added to 5 x 10^6 primed syngeneic WS for in vitro culture as detailed in Methods. WS controls were 19,440 and 4400 PFC/culture in experiments 1 and 2, respectively.

TABLE 2

BMSC ACTIVITY IN COMMON STRAINS OF INBRED MICE

Strain	Haplotype	BMC Added[1]	PFC/Culture	%Suppression
C57B16	H-2^b	-	1520	—
		+	200	87
Balb/cJ	H-2^d	-	2120	—
		+	613	71
DBA/2	H-2^d	-	907	—
		+	253	72
AKR	H-2^k	-	1693	—
		+	720	57
B10.A(3R)	H-$2^{d/b/k}$	-	2147	—
		+	800	63
B10.A(4R)	H-$2^{k/b}$	-	2413	—
		+	573	76
B10.S	H-2^s	-	3133	—
		+	1760	44
A.TH	H-$2^{s/d}$	-	533	—
		+	253	53
CBA/J	H-2^k	-	3693	—
		+	453	88
(B6 X D2)F_1	H-$2^{b/d}$	-	1453	—
		+	467	68
(B6 X A)F_1	H-$2^{b/a}$	-	2533	—
		+	0	100
(B6/TL$^+$ X A)F_1	H-$2^{b/a}$	-	3920	—
		+	547	86

1. 5 x 10^6 WS cultured alone (-) or with 3 x 10^6 syngeneic BMC (+) as detailed in Methods.

TABLE 3

THE BMSC DOES NOT HAVE ANY STANDARD T-LYMPHOCYTE SURFACE ANTIGENS[1]

Treatment of BMC Added[2]	Experiment[3] 1	2	3	4	5	6	7
None added	2893	2027	2253	3920	4000	3040	1840
Untreated	320 (89)	840 (59)	1013 (55)	867 (78)	27 (99)	1760 (42)	107 (94)
Complement (C)	267 (91)	773 (62)	867 (62)	240 (94)	13 (100)	173 (96)	40 (98)
α - Thy 1.2 + C	280 (90)	700 (65)					27 (99)
α - Ly 1.2 + C	160 (94)						
α - Ly 2.2 + C	240 (92)		987 (56)		0 (100)		
α - BAT + C		600 (70)					
α - Qa 1 + C				200 (95)			
α - Qa 2/3 + C				840 (79)			
α - I-J + C			867 (62)				
α - Ly 5.1 + C					27 (99)		
α - N.K. + C					13 (100)		
α - H-2^k + C						2707 (11)	1320 (28)

1. Results expressed as PFC/culture (% suppression).

2. After anti-sera treatment indicated, 3×10^6 BMC (or an equivalent post-treatment number - see Methods) were added to 5×10^6 WS for *in vitro* culture as detailed in Methods.

3. The mice used in experiments 1 - 7 were Balb/c, C57B16, C57B16, (B6/TL^+ X A)F_1, C57B16, B10.S, and SJL, respectively.

TABLE 4

BONE MARROW SUPPRESSION IS PRESENT IN MICE LACKING T-CELLS

Experiment	BMC Added[1]	PFC/Culture	%Suppression
	none	400	
I	Balb/ByJ	53	87
	Balb/ByJ nude	13	97
	none	2533	
II	$B6AF_1$	0	100
	$B6AF_1$ "B"[2]	0	100

1. 3×10^6 BMC added to 5×10^6 WS. Spleen cells from Balb/ByJ and $B6AF_1$ mice in experiments I and II, respectively. See Methods.
2. $B6AF_1$ "B" mice depleted of T-cells by thymectomy followed by lethal irradiation and reconstitution with anti-theta + RC treated syngeneic BMC. See Methods.

TABLE 5

THYMOPOIETIN DOES NOT INDUCE THY 1.2 SURFACE ON THE BMSC

Experiment	Concentration of Thymopoietin (μg/ml)[1]	PFC/Culture		
		WS Only	Treatment of BMC Added	
			C	α-Thy1.2 + C
I	1.0	1880	0	0
	0.1		0	0
	0.01		0	0
	0.001		0	0
II	1.0	2240	13	13
	0.1		293	13
	0.01		27	13
	0.001		40	0

1. Thymopoietin (TP) was diluted in RPMI 1% FCS to the concentrations indicated BMC were then incubated in one of the solutions for 2 hrs. at 37°C, thoroughly washed, counted, and subsequently treated with either complement alone (C) or α -Thy 1.2 + C before being added to WS for in vitro culture (see Methods). All cells were obtained from C57B16 mice.

TABLE 6

THE BMSC DOES NOT EXPRESS SURFACE Ig

BMC Added[1]	Experiments[3] 1	2	3	4
None	2387	2253		
Unseparated	13 (100)	1320 (41)		
Ig^+	5760 (0)	3120 (0)		
Ig^-	120 (95)	1013 (55)		
None			1587	2100
Balb/c[2]			613 (61)	320 (85)
u - suppressed Balb/c			200 (87)	293 (86)

1. BMC from C57B16 mice were separated according to their adherence (Ig^+) or non-adherence (Ig^-) to plates coated with goat anti-mouse Ig (see Methods) and subsequently added to syngeneic WS for in vitro cultures as detailed in Methods.
2. BMC from Balb/c mice that had been injected from birth with rabbit anti-mouse Ig so as to prevent the generation of sIg^+ cells (see Methods) or control littermates injected with normal rabbit serum were cultured in vitro with WS obtained from other Balb/c mice as detailed in Methods.
3. Values represent PFC/culture (% suppression).

TABLE 7

THE BMSC DOES NOT POSSESS AN F_c RECEPTOR

BMC Added[1]				
Unseparated[2]	F_c Adherent	F_c Non-adherent	WS	PFC/Culture
-	-	-	+	2907
+	-	-	+	480
-	+	-	+	2120
-	-	+	+	667

1. C57B16 BMC added to 5 x 10^6 syngeneic WS for <u>in vitro</u> culture as detailed in Methods. BMC added was 3 x 10^6 (unseparated BMC) or its equivalent (F_c adherent or F_c non-adherent BMC) as explained in Methods.

2. Cells harvested from petri dishes covered with anti-SRBC Ig coated SRBC's. Adherent cells are F_c receptor positive while non-adherent cells lack the F_c receptor (see Methods).

TABLE 8

THE BMSC DOES NOT REQUIRE Ly23$^+$ CELLS TO EXPRESS SUPPRESSION

Experiment	BMC Added	BMC Treatment[1]	Responding Spleen Treatment[2]	PFC/Culture (%Suppression)
	None	None	None	2893
	Balb/c	None	"	293 (90)
	"	*a*-Thy1 + C	"	253 (91)
I	"	*a*-Ly23 + C	"	573 (80)
	None	None	*a*-Ly23 + C	3960
	Balb/c	None	"	280 (93)
	"	*a*-Thy1 + C	"	147 (96)
	"	*a*- Ly23 + C	"	67 (98)
	None	None	None	4400
	None	"	*a*-Ly23 + C	8053
	C57B16	"	None	1547 (65)
II	"	"	*a*- Ly23 + C	200 (98)
	DBA	"	None	1240 (72)
	"	"	*a*- Ly23 + C	1493 (81)

1. 3×10^6 BMC added to 5×10^6 WS after the indicated antisera treatments of the BMC (see Methods).

2. WS treated with the indicated antisera before being used for in vitro cultures. WS obtained from Balb/c and C57B16 mice in experiments I and II, respectively.

TABLE 9

THE BMSC DOES NOT SECRETE A SOLUBLE SUPPRESSOR FACTOR

Experiment	BMC Added[1]	Conditioned Supernatant Added[2]	PFC/Culture
I	None	None	4220
	3×10^6	"	520
	None	2 Day	3360
	"	3 Day	4680
	"	4 Day	3060
	"	5 Day	3840
II	None	None	2387
	3×10^6	"	120
	None	1 Day	3240

1. BMC or BMC-conditioned media added to 5×10^6 syngeneic WS for *in vitro* culture as detailed in Methods. Mice used in experiments I and II were Balb/c and C57Bl6, respectively.

2. BMC-conditioned supernatants (see Methods) were extracted from cultures of the indicated ages and subsequently used for WS culture media in a 1:1 ratio with the usual culture media. The secondary culture involved only WS and the usual SRBC concentration to elicit anti-SRBC PFC's.

TABLE 10

BMSC ACTIVITY IS NOT RESTRICTED BY Mls, V KAPPA, V_H, OR BACKGROUND GENETIC DIFFERENCES

Experiment	BMC Added[1]	WS Added	PFC/Culture	% Suppression
1)	None	CBA/CaJ	6360	
	CBA/CaJ (Mls^b)	"	1360	79
	CBA/J (Mls^d)	"	800	87
2)	None	C57B16	8747	
	C57B16 (Ly1.2,2.2,3.2)	"	307	96
	M 16 (Ly1.2,2.1,3.1)	"	120	99
	None	M 16	7067	
	M 16	"	133	98
	C57B16	"	173	98
3)	None	Balb/c	1867	
	Balb/c (Igl^a)	"	493	74
	CB.20 (Igl^b)	"	147	92
	None	CB.20	1000	92
	Balb/c	"	80	96
	CB.20	"	40	
4)	None	DBA/2	3840	
	DBA/2 ($H\text{-}2^d$,Mls^a)	"	1160	70
	NZB ($H\text{-}2^d$,Mls^a)	"	1947	50

1. In vitro cultures using 3×10^6 BMC and 5×10^6 WS were prepared and assayed as noted in Methods. The mouse strains in experiments 1-3 are known to possess genetic differences at the loci indicated in parentheses, while the mice in experiment 4 have dissimilar background genomes and identical H-2 and Mls loci.

TABLE 11

BMSC ACTIVITY IS NOT H-2 RESTRICTED

Experiment	BMC Added (haplotype)[1]	WS Added (haplotype)	PFC/Culture	(% Suppression)
1)	None	C57B16 (H-2^b)	1347	
	Balb/c (H-2^d)	"	387	(72)
	CB.20 (H-2^d)	"	253	(81)
2)	None	C57B16 (H-2^b)	4400	
	DBA/2 (H-2^d)	"	1240	(72)
3)	None	CBA/CaJ (H-2^k)	4120	
	C57B16 (H-2^b)	"	240	(94)

1. In vitro cultures using 3×10^6 BMC and 5×10^6 WS were prepared and assayed as detailed in Methods. BMC and WS were obtained from mice mismatched at H-2, with the relevent alleles indicated in parentheses.

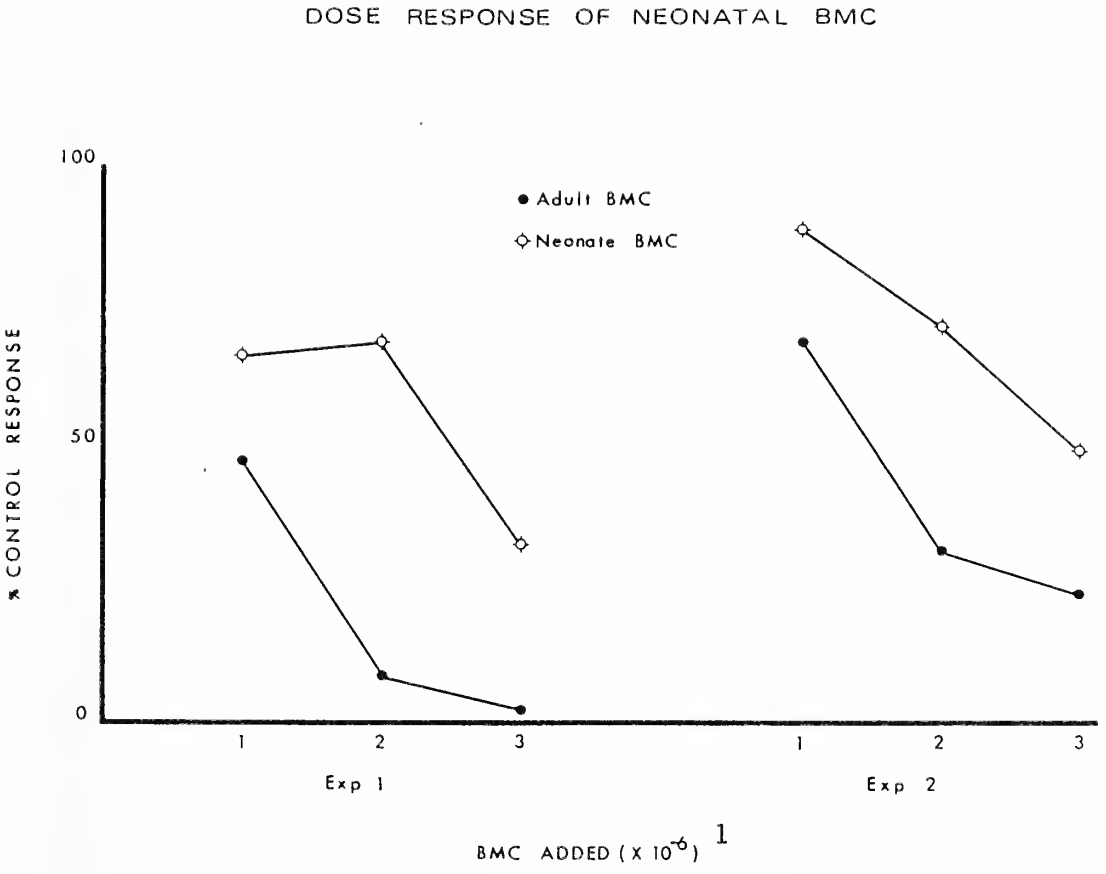
TABLE 12

NEONATAL BM IS LESS SUPPRESSIVE THAN ADULT BM

Experiment	BMC Added[1]	#BMC/Neonate[2]	PFC/Culture	%Suppression
1)	None		1520	
	Adult		200	87
	Neonate (7-9 d)	9.7×10^6	1720	0
2)	None		1800	
	Adult		0	100
	Neonate (10 d)	12.5×10^6	2820	0
3)	None		2413	
	Adult		573	76
	Neonate (11-13 d)	19.2×10^6	1453	40

1. BMC obtained from tibias and femurs of all adult and neonatal mice. All adult mice were at least 8 weeks old while neonates were of the ages indicated in parentheses (in days). 3×10^6 BMC were cultured *in vitro* with 5×10^6 syngeneic WS as detailed in Methods. Mice used were C57B16, C57B16, and B10.4R in experiments 1, 2, and 3, respectively.
2. The pooled mean #BMC/mouse efter flushing BM from femurs and tibias. Numbers of neonatal mice used were 29, 8 and 15, in experiments 1, 2, and 3, respectively.

FIGURE 2



1. C57B16 BMC added to 5 x 10^6 syngeneic WS for in vitro culture as detailed in Methods. WS controls were 19,440 and 3120 PFC/culture in experiments 1 and 2, respectively.

TABLE 13

NEONATAL BM CONTAINS A Thy 1^+ CELL THAT REDUCES BMSC ACTIVITY

BMC Added[1]	BMC Treatment	PFC/Culture (% Suppression) Exp. 1	Exp. 2	Exp. 3
None		1680	4030	1814
3×10^6	None	1213 (28)	3107 (33)	1387 (24)
3×10^6	Complement (C)	1293 (23)	1820 (55)	1080 (40)
3×10^6	α-Thy 1.2 + C	880 (48)	973 (76)	760 (58)

1. BMC added to 5×10^6 syngeneic WS for in vitro culture (see Methods). BMC used after antisera treatments were added in numbers equivalent to the complement controls as discussed in Methods. Mouse strains used were C57B16, B10.1R, and B10.3R, in experiments 1, 2, and 3, respectively.

TABLE 14

NEONATAL BM CAN REDUCE ADULT BM SUPPRESSION

Experiment	Total BMC Added[1]	%Neonatal BMC[2]	% Suppression
I	None	-	WS control-1814 PFC/cult.
	3×10^6	0	56%
	4×10^6	25	43%
	3×10^6	100	24%
II	None	-	WS control-2413 PFC/cult.
	3×10^6	0	76%
	4×10^6	25	55%
	3×10^6	30	51%
	4×10^6	50	59%
	3×10^6	100	40%

1. BMC added to 5×10^6 syngeneic WS for *in vitro* culture (see Methods). Mouse strains used were B10.3R and B10.4R in experiments I and II, respectively.
2. The fraction of total BMC added that came from neonatal mice.

TABLE 15

CERTAIN CBA SUB-STRAINS HAVE LOW LEVELS OF BM SUPPRESSION AS ADULTS

Experiment	BMC Added[1]	PFC/Culture	%Suppression
1)	None	1206	
	CBA/J	320	73
	CBA/CaJ	960	20
2)	None	7380	
	CBA/J	1280	83
	CBA/H	7200	2
3)	None	2067	
	CBA/J	453	78
	CBA/N (homozygous)[2]	1907	8
	CBA/N (defective F_1)	2013	3
	CBA/N (nondefective F_1)	2093	0

1. 3×10^6 BMC added to 5×10^6 WS for *in vitro* culture (see Methods). WS used was CBA/CaJ, CBA/CaJ, and CBA/N (nondefective F_1) in experiments 1, 2, and 3, respectively.
2. Homozygous CBA/N females mated with C57B16 males to produce defective (male) and nondefective (female) F_1 heterozygotes.

TABLE 16

ADULT BM WITH LOW SUPPRESSION HAS A Thy 1^+ BM CELL THAT REDUCES BM SUPPRESSION

Experiment	BMC Added[1]	BMC Treatment[2]	PFC/Culture (%Suppression)
1)	None	-	680
	CBA/CaJ	None	1080 (0)
	"	Complement (C)	707 (4)
	"	α-Thy1.2 + C	307 (55)
2)	None	-	7380
	CBA/H	None	7200 (2)
	"	C	2160 (71)
	"	α-Thy1.2 + C	649 (91)
3)	None	-	2067
	CBA/N (nondefective F_1)	None	2013 (3)
	"	C	2267 (0)
	"	α-Thy1.2 + C	1307 (37)

1. 3×10^6 BMC added to 5×10^6 WS for *in vitro* culture (see Methods). WS used in experiments 1, 2, and 3 were CBA/J, CBA/CaJ, and CBA/N (nondefective F_1), respectively.
2. Complement used in antisera treatments was "low tox" (see Methods) in experiments 1 and 3, while guinea pig complement was used in experiment 2.

TABLE 17

THE BMC FRACTION ADHERENT TO PLATES COATED WITH THE LECTIN V. VILLOSA IS ENRICHED FOR CONTRASUPPRESSOR ACTIVITY

Experiment	BMC Added[1]	Spleen Cells Added[2]	PFC/Culture
I	None	WS	1720
	Unseparated	WS	1280
	Non-adherent	WS	680
	Adherent	WS	2200
II	None	WS	4120
	Unseparated	WS	1080
	Non-adherent	WS	1240
	Adherent	WS	4600
	Adherent, treated (α-Thy1.2 + C)	WS	1600
	None	B Cells	0
	Non-adherent	B Cells	0
	Adherent	B Cells	0
	None	B Cells + T Cells	8160

1. 3×10^6 BMC (or its equivalent) were added to 5×10^6 syngeneic WS or B cells as detailed in Methods. BMC were fractionated according to their adherence or non-adherence to plates coated with the lectin V. Villosa (see Methods). Mice used were neonatal C57B16 and adult CBA/CaJ, in experiments I and II, respectively.
2. B cells and T cells were fractionated from WS by their adherence (B cells) or non-adherence (T cells) to plates coated with goat anti-mouse Ig (see Methods).

TABLE 18

RABBIT SERUM CONTAINS A FACTOR DIRECTED AGAINST THE MURINE BONE MARROW CONTRASUPPRESSOR CELL

Experiment	BMC Used[1]	Rabbit Complement (RC) Used[2]	% Recovery[3]	PFC/Culture
	None	-	-	3133
	Neonate	None	-	2427
	Neonate	Untreated RC	36%	627
I	Neonate	Heat inactivated RC	41%	1813
	Adult	None	-	1760
	Adult	Untreated RC	22%	173
	Adult	Heat inactivated RC	37%	1267
	None	-	-	7920
	Adult	None	-	2040
II	Adult	Untreated RC	44%	800
	Adult	Heat inactivated RC	75%	1280
	Adult	WS absorbed RC	58%	1000
	Adult	BMC absorbed RC	68%	1720

1. 3×10^6 BMC added to 5×10^6 syngeneic WS for *in vitro* culture as detailed in Methods. Mice used were B10.S (neonates and adults) and CBA/CaJ (adults) in experiments I and II, respectively.
2. BMC were exposed to normal rabbit serum (RC), heat inactivated RC (56°C for 30 minutes), or RC absorbed against either WS or BMC, for 30 minutes at 37°C.
3. Fraction of viable BMC remaining after exposure to the RC's indicated.

TABLE 19

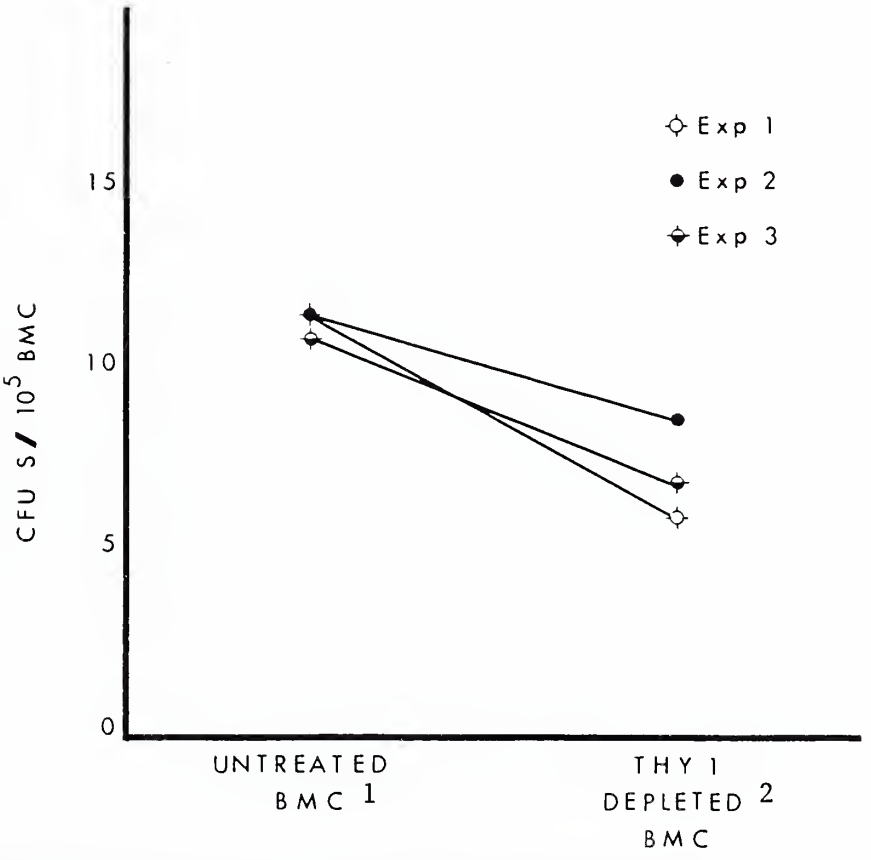
THE RABBIT SERUM ANTI-MOUSE BM ACTIVITY RESIDES IN THE Ig ENRICHED FRACTION

Experiment	BMC Used[1]	GPC[2]	heat inactivated RC[3]	RIg (dilution)[4]	RC Preparation + GPC[5]
1)	CBA/N, F_1	36%[6]	31%		65%
2)	Neonate	34%			66%
	Adult	34%			52%
3)	CBA/CaJ	11%		17% (1:10)	50%
	CBA/CaJ	11%		9% (1:20)	48%

1. Mice used in experiment 2 were Balb/cByJ. BMC were exposed to the preparations indicated in cytotoxicity assay (see Methods).
2. Guinea pig complement (GPC) used at 37°C for 30 minutes.
3. RC first inactivated by heating to 56°C for 30 minutes, then used as for GPC.
4. The NH_4SO_4 precipitate of rabbit serum resuspended in a minimum volume of PBS then diluted and used as for GPC (see Methods).
5. The rabbit serum (RC) preparation used in each particular experiment was used to resuspend the BMC for 30 minutes at 4°C. The BMC were then washed and exposed to GPC for 30 minutes at 37°C.
6. Results expressed as %cytotoxicity from the treatments indicated.

FIGURE 3

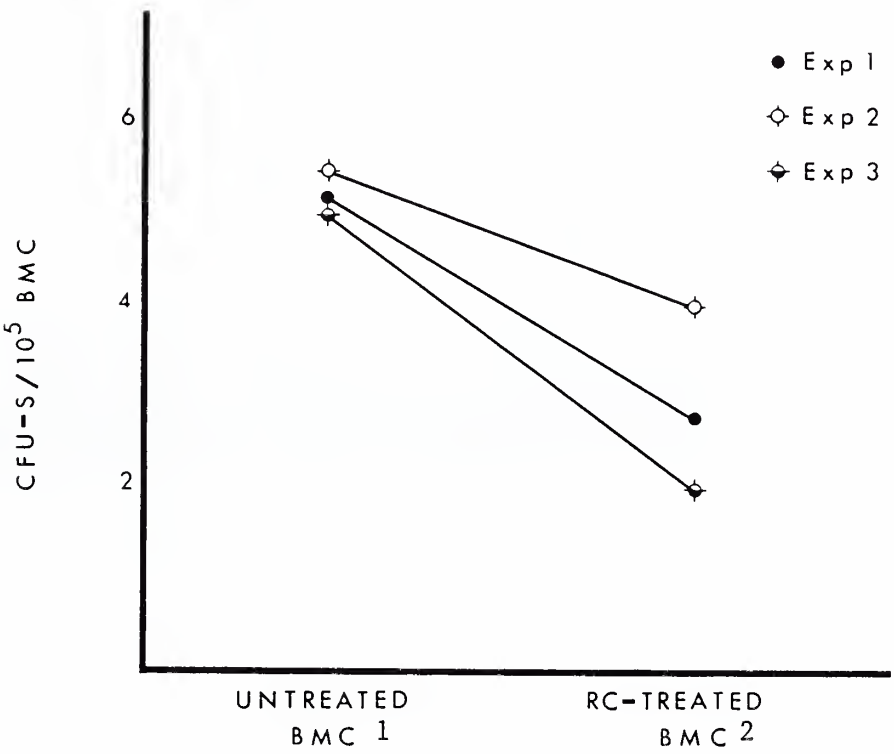
CFU-S EXPRESSION BY BMC IS PARTIALLY THY 1 DEPENDENT



1. 10^5 BMC injected into irradiated syngeneic hosts (see Methods). Mice used in experiments 1,2, and 3 were CBA/N (4/group), CBA/CaJ (4/group), and CBA/CaJ (3/group), respectively.
2. BMC treated with anti Thy1.2 antisera + GPC. The GPC control was 11.0 CFU-S/10^5 BMC in exp.2.

FIGURE 4

RABBIT COMPLEMENT TREATMENT REDUCES BMC-DERIVED CFU-S



1. BMC injected into irradiated syngeneic hosts (see Methods). Mice used in experiments 1,2 and 3 were $C_2D_3F_1$ (2/group, 10^6 BMC injected), BDF_1 (2/group, 10^5 BMC injected), and CBA/CaJ (2/group, 5 x 10^4 BMC injected), respectively.
2. BMC exposed to RC for 30 minutes at 37°C before injection.

TABLE 20

RABBIT SERUM TREATED MURINE BM SUPPRESSES CFU-S EXPRESSION OF UNTREATED MURINE BM

Experiment	BMC Injected[1]		CFU-S/10^5 BMC[2]
	Untreated BM	RC-treated BM	
I	10^6	-	5.2(± 0.2)
	-	10^6	2.8(± 1.5)
	10^6	10^5	2.8(± 0.4)
II	10^5	-	5.5(± 0.2)
	-	10^5	4.0(± 1.5)
	10^5	10^4	1.0(± 0.1)

1. Lethally irradiated mice reconstituted with syngeneic BMC (see Methods). Experiment I used $C_2D_3F_1$ mice and experiment II used BDF_1 mice, with two mice in each group.
2. (± S.E.).

TABLE 21

NEONATAL BM EXPRESSES FEWER CFU-S THAN ADULT BM

Experiment	BMC Injected[1]		CFU/spleen
	Adult BMC	Neonate BMC	
I	3×10^4	-	9.2
	-	3×10^4	3.8*
II	3×10^4	-	9.3
	-	3×10^4	4.0#
	1×10^5	-	16.7
	-	1×10^5	7.0*

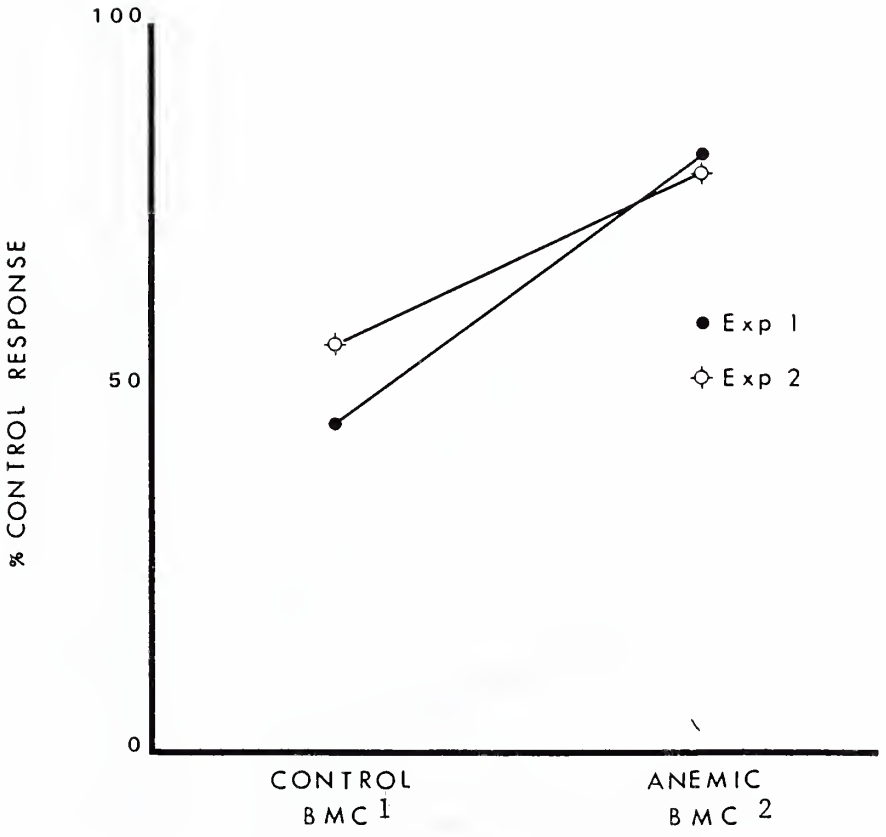
1. Lethally irradiated C57B16 mice reconstituted with syngeneic BMC (see Methods). Each group in experiment I utilized 5 mice, while those in experiment II used 3 mice.

* $P < 0.02$ when compared to similar mice injected with adult BMC.

# $P < 0.04$ when compared to similar mice injected with adult BMC.

FIGURE 5

BMC OF ANEMIC MICE ARE LESS SUPPRESSIVE THAN NORMAL BMC

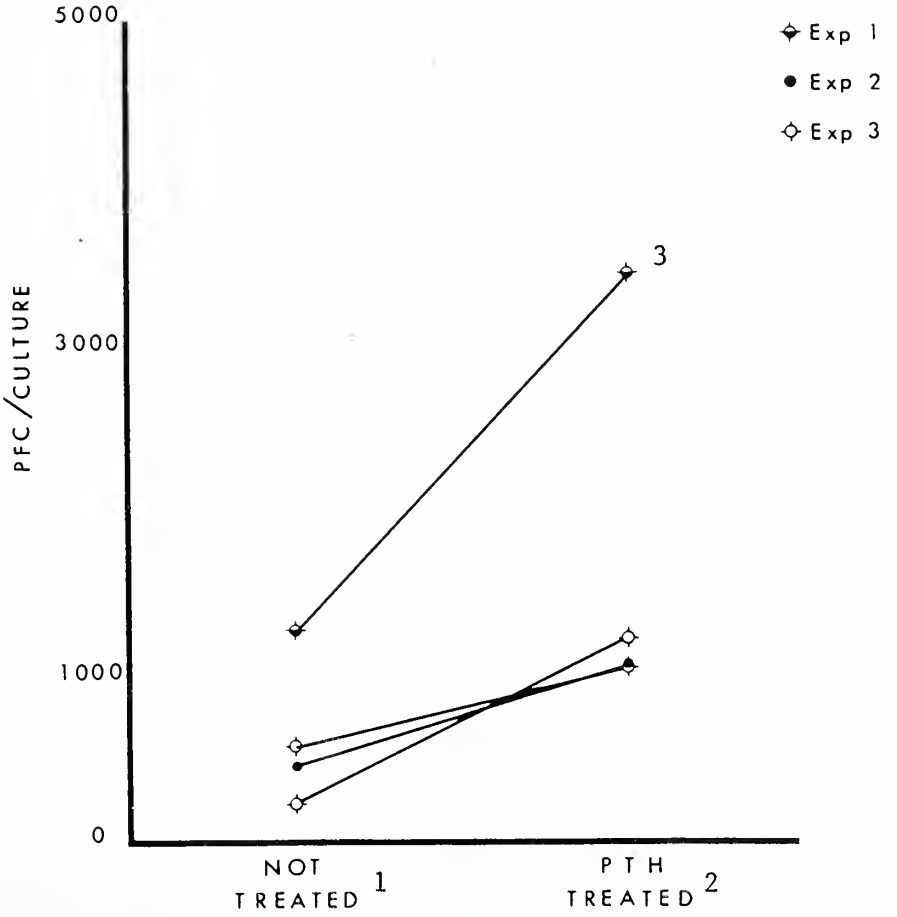


1. 3×10^6 CBA/J BMC added to 5×10^6 (exp. 1) or 10×10^6 (exp. 2) syngeneic WS for *in vitro* culture as detailed in Methods. WS controls were 800 and 2693 PFC/culture in experiments 1 and 2, respectively.

2. Mice made anemic by alternate-day bleeds averaging 0.3 ml. Average hematocrit at sacrifice was 30-35%.

FIGURE 6

PARATHYROID HORMONE MAY ACTIVATE THE CS

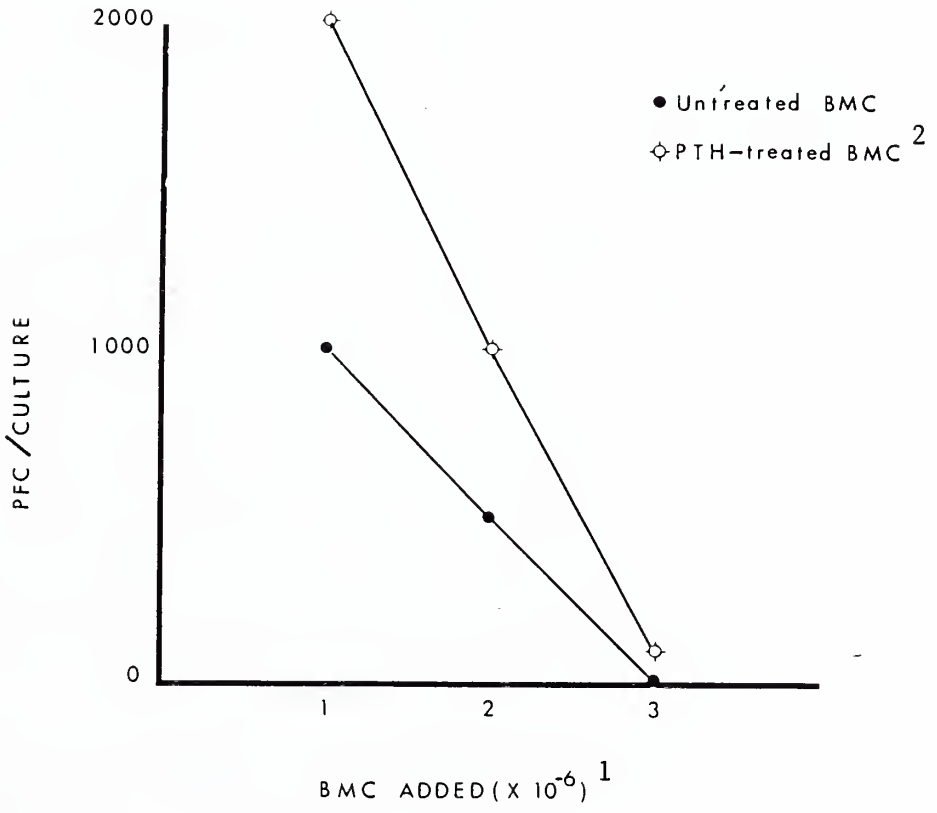


1. 3 x 10^6 BMC added to 5 x 10^6 WS for in vitro culture (see Methods). Experiments 1,2, and 3 involved CBA/J BMC added to CBA/J WS, C57B16 BMC added to C57B16 WS and C57B16 BMC added to CBA/CaJ WS, respectively. C57B16 mice obtained from Charles River Labs (exp. 2 and 2 matched groups in exp. 3) or Jackson Labs (2 matched groups in exp. 3).
2. BMC exposed to PTH before in vitro culture (see Methods).
3. $P < .05$ for paired t-test involving exp. 1 only, exp. 2 and 3 only, or all experiments taken together.

FIGURE 7

DOSE RESPONSE OF BM SUPPRESSION

AFTER PTH TREATMENT



1. C57B16 BMC added to 5 x 10^6 syngeneic WS for in vitro culture as detailed in Methods. WS control was 1800 PFC/culture.

2. BMC exposed to PTH before in vitro culture (see Methods).

DISCUSSION

I. Characterization of the BMSC

It has been appreciated for some time that the bone marrow contains a population of cells that are capable of suppressing an *in vitro* antibody response by lymph node (19) or spleen (18,20,22,23,25,50,51) cells. A definitive characterization of this population has, as yet, eluded investigators, however.

It seems probable that the bone marrow suppressor cell is not a macrophage or other phagocytic cell, as identified by the presence of an Fc receptor (52) or glass adherence (53). All attempts to demonstrate a glass adherent or indomethacin sensitive suppressive activity (data not shown) or to detect an Fc receptor positive BMSC (Table 7) met with failure. With the exception of one report (20), others have also been unable to characterize the BMSC as a glass/plastic adherent (19,25) or carbonyl-ingesting (18,50) cell. Data concerning a BMSC Fc receptor is less compelling, with one report of a Fc receptor positive suppressor cell (18), while another suggested that a subpopulation of BMSC's may be Fc receptor positive although enrichment for suppression was not observed in the Fc receptor positive fraction of BMC (20). Some have inferred from radiosensitivity data, which showed BM suppression to be abrogated by 800-1000 rads of x-irradiation (20,51), that the BMSC was not a macrophage, although one group (22) identified a BM suppressor that was sensitive to as little as 50 rads. Despite a certain amount of conflicting data, the evidence predominately favors the conclusion that the BMSC is not a macrophage.

The possibility of B cell mediated BM suppression was examined by two different techniques. The use of positive selection via Ig plates (Table 6) allowed the observation of sIg^+ and sIg^- cells from the same

pool of BMC simultaneously. The data unequivocally show that the BMSC is sIg^- and, furthermore, that no suppressive activity can be detected in the sIg^+ fraction. Complementary experiments using BMC from u-suppressed mice lacking mature B cells (38) demonstrated that these mice did not have decreased levels of BM suppression. These data, then, do not provide any evidence to support the notion that the BMSC is an sIg^+ B cell. Past efforts to detect sIg on the BMSC have had mixed results, with some unable to demonstrate sIg using anti-Ig columns (18) or anti-Ig antisera + complement treatments (20), while others (19,21) were more successful. The data of Fuch, et al (21) in particular, strongly implies an sIg^+ BMSC and it is difficult to explain the marked discrepancy between their data and that presented here.

The first immunosuppressive cell described (10) was a T cell, as defined by its cell surface antigens (54). That the BMSC might also be a T cell is a natural extension of the suppressor cell concept that was explored. The use of antisera directed at Thy-1 (5), Ly (54), I-J (55), and Qa (56) cell surface antigens to subdivide T lymphocyte populations is a well-established technique that was employed in these investigations. It was abundantly clear (Table 3) that BM suppression was not sensitive to any of these agents. Of some interest was that anti-brain associated theta (BAT) antisera, which has been reported to have specificities for stem cells (57,58), non-T cells (59), pre-T cells (60,61), and some B cells (60,62) in the spleen and BM, was equally ineffective in removing the BMSC. This data is consistent with the observations that fully potent BMC suppression is found in both congenitally athymic nude mice and experimentally produced T depleted athymic "B" mice (Table 4). Attempts to induce Thy-1 on the BMSC by exposing the BMC to thymopoietin, a potent inducer of Thy-1 for precursor T cells (43,44), were also un-

successful (Table 5). Although there is some evidence that differentiation may occur *in vitro* without stimulation (63,64), preculture of BMC for 24 hours neither induced a Thy-1$^+$ BMSC nor reduced overall BM suppression (data not shown). The compelling conclusion is that the BMSC is certainly not a mature T cell and is very unlikely to be a precursor T cell. Other investigators (19,20,22,24), notably Singhal (18,25,50,65), generally using heterologous anti-θ antisera, have similarly concluded that the BMSC was not a T cell, although the possibility of a weakly θ$^+$ cell possessing other T cell markers was not examined. There has been some uncertainty over whether the BMSC is a precursor T cell, with Dauphinee and Talal reporting a BMSC with inducible θ (22) while Corvese, *et al* (20) were unable to find such a cell. The source of discrepancy appears to be the method used for fractionation of BMC. Dauphinee and Talal studied a density gradient fraction that was enriched for lymphocytes while Corvese, *et al* used BMC that were either unfractionated or lymphocyte depleted. In as much as Gorcznski and MacRae (24) showed that the BMSC comigrated with large polymorphonuclear cells during velocity sedimentation and Corvese, *et al* (20) found that suppressive activity was inversely related to the percentage of lymphocytes found in each fraction after free-flow high voltage electrophoresis of BMC. It is likely that the suppressor cell of Daupinee and Talal is a low-θ classical suppressor cell that migrated to the BM. That such peripheral blood lymphocytes can reside in the BM is well known (66).

It was also found that the BMSC did not bear surface markers that are associated with natural killer cells found in the BM (49). This was the first demonstration that the BMSC is Ly-5$^-$ and N.K.$^-$ (Table 3), although some hint of this is contained in earlier work.

Natural killers require the presence of a viable BM (49) and they can be shown to decrease in activity after ^{89}Sr treatment to obliterate the BM (49,67). Of note is the observation (49,67) that under these conditions, a non-T, sIg^-, BAT^- splenic suppressor cell emerges in inverse proportion to the decrease in natural killer cell activity. This suggests that not only is the BMSC not a natural killer cell, but it also appears capable of migrating out of the BM cavity when stressed.

Although the BMSC at present travels somewhat incognito, experimental evidence presented here defines the effector arm of BM suppression. It is clear from Table 8 that BM suppression does not require the presence of a classical suppressor cell, as the complete elimination of $Ly\text{-}2,3^+$ cells from the *in vitro* culture system does not abrogate suppression. This implies that the BMSC acts on either the T helper cell or B cell directly. That the BMSC effectively suppresses a secondary WS response indicates that the BMSC acts to suppress immunocompetent cells rather than to prevent the development of an antibody response. This is in full agreement with Duwe and Singhal (23,65), who demonstrated BM suppression of B cell cultures supplemented with T cell factors, and Corvese, *et al* (20), who showed that BM was equally suppressive for responses to T-dependent of T-independent stimuli. Thus it would seem that the BMSC suppresses B cells directly.

It is likely that the suppressive activity of BMC requires cell-to-cell contact. This conclusion stems from Table 9, which demonstrates that the BMSC does not secrete a stable soluble suppressor factor. Although no clear contradiction to this result has been reported (19,51) there is some controversy over experiments involving double-chamber diffusion cultures in which the BMC are separated from the responding

lymphocytes by a membrane. Using a membrane of 0.2u pore size, Duwe and Singhal (65) were able to generate dose-dependent BMC suppression. In contrast, Petrov, et al (68) were unable to demonstrate a soluble suppressor factor using a partitioning membrane of 25mm pore size even though the membrane was detectably permeable to other secreted BMC factors. This lack of a stable soluble suppressor factor is also another feature that distinguishes the BMSC from the classical T suppressor cell (69).

If the BMC suppression were passive, in the sense that no cellular metabolism was required, it might be expected that heat-killed or mitomycin-C treated BMC would retain their suppressive activity. Although past studies are inconclusive with respect to the suppressive capacity of heat-killed BMC (18,19), Singhal (23,50) maintains that BM suppression is not abrogated by nitomycin-C treatment, a result that is at variance with more recent observations (M. Horowitz, personal communication). It is certainly true, though, that intact BMC are required for suppression to be expressed (25,50).

The failure of the BMSC to conform to the standard T suppressor cell characterizations is perhaps made more understandable by its extraordinary domain of activity. While the induction and action of the classical suppressor cell is V_H and H-2 restricted, respectively (69,70,71), the BMSC is effective regardless of the genetic correspondence of the responding spleen cells. Not only does its activity breach minor histocompatibility barriers, but major histocompatibility (Table 11) and xenogeneic (unpublished observations) differences are also irrelevant. A heretofore unappreciated property of the BMSC, this probably reflects the unique position that BMC occupy within the

hematopoietic system. Although its ontological development is not known, it can be reasonably surmised that, under most circumstances, the BMSC is detectable only in the BM and it probably seeks out the BM cavity preferentially. Under other circumstances, such selective migration of various immunologically active cell populations can be demonstrated (66,67,73,74,75,76), indicating that this can serve as a generalized response to specific stimuli or homeostatic requirements.

What is the function of the BMSC that it finds itself largely limited to the BM cavity? The extensive hematopoietic role of the BM is well known, in general terms at least, but somewhat less appreciated is the degree to which the BM participates in antibody production. It has been demonstrated by Benner (73,74,77,78,79,80), Cudkowicz (81,82,83), and others (72,84,85,86,87), that the BM can serve as a potent source of Ab production. In general, this involves formation and migration of memory B cells, and probably memory T cells, from the spleen to the BM, although at higher antigen doses peripheral lymphoid organs such as lymph nodes and Peyer's patches can replace the staging function of the spleen. The BM contribution to Ab production is minimal during the initial response to antigen, but in succeeding Ag challenges, the BM becomes the predominant source of Ag-specific IgM and IgG (77,79). It should be emphasized that this is a result of lymphocyte migration to the BM, since BM probably contains immature or newly mature B cells *in situ* (87) and appears to require T cells for full expression of Ab expression (80,87).

The role of a BMSC in regulating BM Ab production might, therefore, seem to be an obvious one. Such a potent suppressor cell in an important Ab producing organ has an inherent logic about it that is attrac-

tive. Certain inconsistencies must be addressed before this hypothesis is accepted, however. Although the BMSC depresses IgM PFC's more than IgG PFC's (23), the data from those groups studying BM Ab production indicates that not only is IgM and IgG generated in the BM, but the former is usually made in greater quantity (78,83). Duwe and Singhal (18) suggest that the BMSC is responsible for the non-Poisson distribution seen in limiting dilution analysis of BMC PFC's, but this does not explain the Poisson distribution obtained in similar experiments performed by Cudkowicz, et al (81), using BDF_1 mice, which we have shown to possess the BMSC (Table 2). Nor is the question of why IgM PFC's are preferentially suppressed fully considered. It has been speculated (23) that the BMSC could be the effector cell for clonal deletion of autoreactive BM B cells. Although quite alluring, this is incompatible with the results of experiments using neonatal BM (Table 12). It is well known that exposure to Ag in the neonatal period can render the host tolerant to that Ag (88). Consequently, one would expect BM suppression to be, if anything, increased during this period of "immunological inventory-taking". Experiments show, however, that the exact reverse situation was found (Table 12).

II. The phenomenon of BM contrasuppression

The finding of decreased BM suppression in neonatal mice contrasts with the results of Corvese, et al (20), who found BM of 14 day old mice to have adult levels of suppressive activity. This discrepancy is almost certainly due to the relatively advanced age of the mice used by Corvese, et al as a sharp increase in BM suppression at about day 12 of life can be appreciated in Table 12. Furthermore, the reduced BM suppression can be attributed to the presence of a Thy-1^+ cell that interferes with the expression of suppression by the BMSC.

Not only does the removal of this cell, the BM contrasuppressor cell, increase neonatal BM suppression (Table 13), but also the addition of neonatal BMC to highly suppressed adult BMC serves to reduce the suppression manifested by the adult BMC. The latter phenomenon also partially answers yet another question, that of the ontogeny of BM suppression. Theoretically, the development of BM suppression can be viewed as the outcome of one of three possible processes: a) a decline in the activity of the contrasuppressor, b) a shift in the balance of numbers between the BMSC and the contrasuppressor, or c) a developed resistance of the BMSC to the contrasuppressor effect. That neonatal BMC can act as a source of contrasuppression to reduce adult BM suppression implies that adult BM suppression cannot be completely ascribed to BMSC insensitivity to the effector arm of contrasuppression.

To be sure, this does not identify the effector arm of contrasuppression on a cellular level. A recently identified cell with similar actions (15,16) has been elegantly shown to make T helper cells resistent to suppression. Although this demonstration involved quite different cell populations of unexplored homology to the BM populations, this other contrasuppressor was a T cell that was selectively adherent to the lectin Vicia Villosa (41), making it similar to the BM contrasuppressor (Table 17). Thus it is quite possible, if not likely, that similar cells and cellular mechanisms are operating in both experimental situations.

The discovery of decreased suppression among certain CBA substrains was unexpected. Given the phenotypic identity between the neonatal and CBA contrasuppressors (e.g. both are Thy-1$^+$ and selectively adherent to Vicia Villosa), it is reasonable to consider them to be identical cells.

The only difference between them is that the CBA contrasuppressor is present in adult mice, presumably a result of a particular genetic endowment (see Appendix II). It has been previously appreciated that there are subtle immunogenetic differences between CBA/J and CBA/H mice, some of which relate to T-independent antigen responsiveness (M. Iverson, personal communication). While this by no means constitutes proof, it does suggest that inquiry based upon suitable recombinant mice might provide a genetic locus for BM suppression.

In the course of experiments, it was found that exposure of BMC to rabbit complement resulted in greater suppression. Although it was usually of little importance in experiments concerned with the BMSC, it became a major obstacle in the phenotyping of the contrasuppressor. The data in Table 18 give representative examples of the loss of contrasuppression caused by rabbit complement. This was seen with all rabbit complements used, including commercially available rabbit complement screened for extremely low background lymphocyte cytotoxicity, but was less evident for guinea pig complement. Interestingly, similar data has been published by others without comment (20). This precluded further phenotyping of the contrasuppressor, since all available antisera directed against Ly, I-J, and Qa cell-surface antigens were only compatible with rabbit complement.

Table 18 also demonstrates that while the anti-contrasuppressor activity in RC is somewhat relieved by heating inactivating the RC, it was also removed by absorption of the RC with BMC and, to a lesser extent, WS syngeneic to the mouse strain used. This suggestion of antigen specificity is further explored in Table 19, which shows that RC possesses an antibody-like molecule that is directed against BMC.

It is likely that this represents heterogeneous immunoglobulin, similar in nature to well-described heterophile antibodies seen elsewhere (89), with specificities that include the BM contrasuppressor.

III. The regulation of hematopoiesis

Another possibility for the role of the BM regulatory circuit is in the control of hematopoiesis. In this regard are intriguing reports that the spleens of mice treated with ^{89}Sr, which selectively obliterates the BM compartment, possess a splenic suppressor cell that is Thy-1$^-$, BAT$^-$, sIg$^-$, and sensitive to 1000 rads of irradiation (64,67). Such mice develop extramedullary hematopoiesis and appear to be similar to osteopetrotic mice, whose BM cavity is obliterated by a genetically transmitted myelofibrosis and who also express splenic extramedullary hematopoiesis in association with a Thy-1$^-$ splenic suppressor cell (H. Cantor, personal communication).

In such a role, a decrease in BM suppression would be expected under conditions of hematologic stress requiring increased BM production of blood constituents. The neonatal mouse is in just such a situation, as its rapid growth necessitates a concomitant surge in hematopoiesis. Data presented here shows that under these conditions BM suppression is drastically reduced (Table 12). Closer examination reveals that once the neonate generates more BMC by days 11 to 13, BM suppression increases, as would be expected in a regulatory response to prevent uncontrolled proliferation. Experiments addressing the question of hematpoiesis in CBA mice with high adult levels of contrasuppression have been undertaken but time constraints have limited the amount of data obtained.

A series of experiments utilizing the splenic colony forming units (CFU-S) assay as a model of hematopoiesis was undertaken to

elucidate the regulatory role of BM contrasuppression. Before discussing the results, a brief description of the CFU-S assay, its physiology, and its limitations are warranted.

The CFU-S assay is quite simple (45). Syngeneic BMC are injected intravenously into lethally irradiated mice, who are subsequently sacrificed 10 days later and their spleens examined for macroscopic nodules, each of which represents a hematopoietic CFU arising from a single pluripotent stem cell (90,91). These stem cells, which are not morphologically distinctive (46,92) have the capacity to generate either myeloid or erythroid CFU's. Although control of differentiation can be theoretically attributed to random events, long range factors (i.e. hormones), and/or local microenvironmental factors (91,93), the last possibility has been clearly shown to be influential (46,94,95, 96,97). In general, CFU-S tend to be predominantly erythroid (96,97).

It would be predicted that if the contrasuppressor participated in hematopoietic regulation, then removal of the contrasuppressor might result in fewer BMC-derived CFU-S. Figure 3 shows that in 3 experiments using CBA/N or CBA/CaJ mice, reduction of contrasuppression by treatment of the BMC to remove Thy-1^+ cells resulted in up to a 50% decrease in CFU-S production. Work by others has generally demonstrated reduced CFU-S recoverable from BM of neonatally thymectomized mice (28,98,99). This condition could be corrected by the implantation of either a thymus graft (28) or diffusion chamber containing thymic tissue (27), although infusion of thymocytes was without effect (28) and congenitally athymic mice have been reported to possess a normal number of CFU-S/10^5 BMC (100,101). The infusion of parental thymocytes into an irradiated F_1 recipient of parental BMC has been shown to increase the number of

CFU-S (29), probably secondary to improved seeding efficiency of BMC, into the spleen (102), as well as induce an increase in the size of CFU-S's (30). In other experiments, thymocytes caused a shift in the erythroid:myeloid ratio towards increased myeloid colonies (31,32,103), while in one particularly complex experiment, the presence of host T cells appeared to be required for the generation of CFU-S (32). Despite such evidence for thymocyte influence in the CFU-S assay, a role for an endogenous Thy-1^+ BMC in CFU-S expression has not previously been demonstrated.

In other models of hematopoiesis, T cells have been shown to augment erythropoiesis (33,104) and neonatal thymectomy in opossums has been noted to result in maturational arrest in the erythroid cell line (105). By examining colony forming units grown in in vitro agar cultures (CFU-C), a family of glycoproteins necessary for culture, called colony stimulating factors or activities (CSF, CSA), has been discovered. These CSF's are usually derived from T lymphocytes (106), unfractionated spleen cells (107,108,109) or miscellaneous tissues (94,110), but their generation appears to be T cell dependent regardless of the tissue being used for production (106,109). Despite the fact that CFU-C are predominantely myeloid and quite different from CFU-S, it is of some interest that CSF has been reported to have unusual effects upon CBA/H-derived CFU-C. In CBA/H mice, not only do their CFU-C contain an uncommonly large erythroid component (109,111), but their BMC are much more sensitive to the trophic effects of CSF than other mice (111). In light of the heightened contrasuppressive characteristics of CBA/H mice, these observations are consistent with the concept of a role for contrasuppression in the regulation

of differentiation of hematopoietic stem cells.

In another intensively studied system, the genetically transmitted anemia seen in W/W^v mice has been shown to be amenable to cure by the adoptive transfer of normal BM with a source of donor T cells (either the untreated normal BM or normal thymocytes) (112,113). It is not clear how this relates to T cell influence over CFUS expression, since these reports maintain that the number of CFU-S is not altered by anti-theta + complement treatment of BMC, while one (112) shows that such treatment of BMC actually increases the proportion of myeloid colonies - opposite of what would be expected based on other observations (31, 32,103). These data have been interpreted as demonstrating the existence of a stem-cell independent, theta-sensitive regulatory cell (TRSC) that regulates stem cell differentiation (112,113).

Similar conclusions have been advanced concerning thymocyte influence on endogenous CFU-S in sublethally irradiated mice (114) and the role of rabbit anti-mouse brain (RAMB) antiserum in the removal of CFU-S activity from BMC (115). This last observation is particularly interesting, as Poverenny, et al, based their conclusions upon their ability to partially restore CFU-S after RAMB treatment by injecting semi-syngeneic thymocytes, syngeneic thymocytes, or thymosin Fraction V into the host. In the absence of absorption specificity controls for the RAMB, as was provided by Golub in his original report (116), the data could also be explained by another property of the RAMB, that being the endogenous anti-contrasuppressor activity found in rabbit serum. Lastly, Tyan (117) presents data that he interprets as giving evidence for a somewhat elaborate regulation of CFU-S proliferation. In addition to the

use of a rather contrived experimental design involving immunization of donor mice against SRBC, Tyan fails to use the appropriate complement controls those experiments in which BMC are treated with anti-Ly antisera plus RC. In those experiments, Tyan used a GPC control, a clearly inappropriate maneuver in light of the data presented here.

If contrasuppressor regulation of CFU-S is to be hypothesized, it was necessary to first demonstrate the existence of suppressive control of CFU-S expression. Table 20 deals with this question by examining the effect of adding RC-treated BMC - to normal BCM. In both experiments, such a mixture resulted in decreased CFU-S compared to untreated BMC. Although the small number of mice used in these experiments precludes any firm conclusions from being drawn, it did appear that RC treatment of BMC unmasked a suppressive activity directed at CFU-S expression. These experiments also indicate that the CFU-S reducing activity of RC is not primarily due to an anti-stem cell activity, although minor losses of stem cells cannot be ruled out. Consequently, the RC activity can be said to be directed against a cell that reduces suppression, a contrasuppressor possibily analogous to Poverenny's CFU-S accessory cell (115), in the CFU-S assay system.

In all the experiments discussed, the perceived connection between the in vitro culture and CFU-S assay systems was based upon data derived from manipulations that gave predictable in vitro results with which CFU-S assay results could be compared. Figure 5 turns this process around to demonstrate that physiologic stressing of BM hematopoiesis can result in alterations of the in vitro culture characteristics of BMC. In these experiments, the BM of anemic mice was much less suppres-

sive than that of control mice. This decrease in suppression could not be attributed to dilution of the BMSC by proliferating hematopoietic cells and, furthermore, suppression was restored towards control levels by depleting the anemic BM of Thy-1$^+$ cells. Previous work has found that anemic mice generate more transferable CFU-S in their BM (118,119) and WS (120,121), and they possess more endogenous CFU-S (120). This is probably a consequence of selectively stressing the erythroid cell line, as bled mice demonstrate increased erythropoietic proliferation (122) and a decreased number of non-erythroid CFU-S (123), while polycythemia results in a decreased number of CFU-S with an increased proportion of non-erythroid CFU-S (123,124). Thus, the data presented here indicates that by stressing BM hematopoiesis in such a way as to increase CFU-S expression, a parallel decrease in in vitro BM suppression is also produced, apparently via increased contrasuppression. At this point, having seen that perterbations in contrasuppression in either experimental system result in parallel changes in the other, and given the phenotypic correspondence between the contrasuppressors in either system, it is not unreasonable to hypothesize that the two contrasuppressors are very closely related cells, perhaps even identical.

There is one piece of data that seemingly contradicts the above discussion, however. In Table 21, two different experiments utilizing three separate experimental groupings clearly show that neonatal BMC generates significantly fewer CFU-S than adult control BMC. Given the neonate's severe hematopoietic stress and well-documented high level of contrasuppression, this data obscures the relationship between CFU-S, physiologic hematopoiesis, and contrasuppression. This might be explained by the presence of an artifactual factor in the CFU-S assay; however. It

is easily appreciated that the initial controlling event in CFU-S generation is the lodging of the stem cell in the recipient spleen. The seeding efficiency, the percentage of stem cells successfully lodging in the spleen, has been estimated to be 10-15% under normal conditions (31,125). This figure, though, has been shown to be sensitive to the proliferative activity of the cells used. In particular, during fetal and neonatal life, hematopoietic cells have extremely short cycling times (122,125), a condition that is associated with a seeding efficiency only 1/3 to 1/2 of normal (125). It is quite likely, then, that the reduced CFU-S seen with neonatal BMC is due to an artifact of the CFU-S assay rather than to a lack of contrasuppressive influence over hematopoiesis.

If the Thy-1$^+$ contrasuppressor is, in fact, a significant factor in the control of in vivo hematopoiesis, it might then be possible to link some clinical hematological disorders to derrangements in suppressor-contrasuppressor balance. Of particular relevance are the numerous studies of various types of BM failure resulting in anemia, neutropenia, or pancytopenia. In Diamond-Blackfan Syndrome, the pure red cell hypoplasia has been associated by some investigators to lymphocytic suppression of BM (126), but this view is not unanamous (127,128). Acquired red cell aplasia can sometimes be ascribed to circulating autoantibodies (129), but good evidence for a cell mediated immunologic dysfunction is lacking (128). In neutropenia associated with collagen vascular diseases, particularly Felty's syndrome, it has been observed that suppression of myeloid CFU-C is T lymphocyte dependent (130,131). In isolated neutropenia, there appears to be a non-T mononuclear cell in BM and peripheral

blood that also suppresses CFU-C expression (132,133). Comparable studies of aplastic anemia have shown that up to 50% of patients have a mononuclear cell (134) or non-T lymphocyte (135) mediated suppression of CFU-C. Interesting, in one report (136), a 12 hour in vitro preculture of the patient's BM greatly augmented its suppressive capability, a phenomenon that has been noted with murine BMC (63,64). From another perspective, the role of contrasuppression in the emergence of hematological neoplasms is a totally unexplored, though intriguing, area of inquiry. On the whole, however, although the literature gives a few tantalizing hints of inappropriate BMSC function in certain disease states, notably isolated neutropenia and some aplastic anemias, no firm conclusions can yet be drawn based on the evidence available.

IV. The regulation of bone metabolism

A guiding principle in the evaluation of the BM contrasuppressor was that its microenvironment was an important determinant of its function. Given its intimate association with bone, a few exploratory experiments were undertaken in an effort to uncover contrasuppressor activity that pertained to bone metabolism.

Normal bone metabolism can be viewed on a cellular level as a dynamic equilibrium in which "the events of formation and resorption are not random, but coupled. Formation follows resorption and takes place at sites of previous resorption" (137). In this interplay, the role of bone resorption is ascribed to the osteoclast (138), a multinucleated cell that has been shown to derive from blood-borne cells (34,139,140, 141,142), possibly mononuclear phagocytes (138,143). Under normal circumstances, the controlling determinant of the level of resorptive activity is parathyroid hormone (PTH), secreted by the parathyroid

glands. PTH increases bone resorption by increasing the activity of existing osteoclasts (138,144) while simultaneously generating an increase in osteoclast numbers by recruitment of blood-borne cells (138,145,146). Interestingly, neither an osteoclast PTH-receptor nor evidence for a direct effect upon monocytes by PTH has yet been discovered, although other bone marrow cells have been shown to possess PTH-receptors (138). This has led to the suggestion that PTH induces local regulatory changes in bone that result in greater monocyte trapping and osteoclast activation (138).

With this in mind, the experiments in Figures 6 and 7, which were designed to examine the in vitro effects of PTH upon BMC, show that the brief exposure of BMC to PTH prior to in vitro culture resulted in significantly less BM suppression. This implies that the level of contrasuppression was increased, although whether this reflects changes in suppression or contrasuppression cannot be discerned from these experiments. It is unlikely that this PTH effect was simply due to dilution of the BMSC by proliferation of unrelated PTH-responsive cells since the PTH exposure time of 3.5 hours was probably too brief to allow significant proliferation compared to the control pre-incubation. Figure 7 supports this contention, in that the divergent slopes of the dose response curves for normal and PTH-treated BMC suppression implies that different cellular interactions were occurring in the two situations. If it were only a matter of BMSC dilution, the curves would be parallel. Thus, either the BMSC or BM contrasuppressor possesses a function PTH-receptor, the activation of which alters the balance between suppression and contrasuppression as detected in an in vitro antibody production assay.

Why an overlap of such disparate biological systems appears to be occurring will be discussed later.

With respect to lymphocyte mediation of bone resorption, much interest has been focused upon the actions of a peculiar lymphokine called osteoclast activating factor (OAF). This lymphokine is produced by activated lymphocytes (138,147), in association with monocytes that secrete prostaglandin E_2 (147,148), and has been implicated as the etiology of the osteolytic lesions seen in multiple myeloma (36,149) and T cell lymphoma (35). Although not completely understood, the biochemical mechanism of action of OAF appears to be different from that of PTH, as indicated by the requirement for an intact prostaglandin pathway for OAF, but not PTH, activity (36,147,148). It is unlikely, though, that the BM contrasuppression phenomenon is prostaglandin mediated since concentrations of PTH up to 100 times that used in these experiments, when used to stimulate in vitro bone resorption, do not give rise to increased prostaglandin synthesis (148).

One experimental model of cellular regulation of bone metabolism has been osteopetrotic rodents that have genetically determined myelofibrosis of the marrow cavity apparent at birth or shortly thereafter. The best known of these animals, the micropthalmic (mi/mi) mouse, has been fruitfully investigated by Walker (139,150,151) and others (141), who have been able to cure the osteopetrotic defect by the adoptive transfer of normal BM or spleen cells to the mi/mi mice. Conversely, the transfer of mi/mi spleen cells to lethally irradiated normal littermates resulted in osteopetrotic recipient mice (141,150). Although the osteopetrotic defect is not T cell in nature, these experiments confirm the cellular nature of the dysfunction. Perhaps more germaine to the

discussion, this data indicates that under these conditions, the cells regulating bone metabolism migrate to the spleen where, it will be recalled, extramedullary hematopoiesis and a Thy-1$^-$ suppressor cell are also found following marrow cavity obliteration (64,67).

Although the data presented here do not conclusively show that PTH increases contrasuppressor (i.e. Thy-1$^+$, RC sensitive) activity, other circumstantial evidence indicates that a Thy-1$^+$ may be involved in the regulation of bone resorption. In a rat model of osteopetrosis, the op/op rat, infantile osteopetrosis was correctable by transplantation of a thymus from a normal littermate (34). In addition, athymic nude mice, who have histologically normal bone structure (34), have been reported to have a much lower bone turnover rate than normal mice (152).

V. The BM contrasuppressor as co-ordinator of BM functions

If it is assumed that the BM contrasuppressor participates in both bone metabolism and hematopoiesis, then it might be possible that these two physiological systems directly interact, especially given their observed co-migration under certain circumstances. A recent report indicates that, at least under extreme physiologic stress, hematopoietic regulatory mechanisms may alter bone metabolism.

It is widely taught that thalassemia major is associated with distinctive skeletal abnormalities. Pootrakul et al (153), found that patients who were severely anemic secondary to thalassemia major also had histologic bone abnormalities consisting of osteopenia and decreased mineralization associated with increased bone turnover and low PTH levels. After simply transfusing these patients to relieve their anemia, the investigators found decreased BM turnover associated with histologic bone changes indicating reduced osteoclastic activity and lower bone turnover, accompanied by an increase in serum PTH. This succession of

events is what would be predicted by a regulatory model involving some degree of interaction between hematopoiesis and bone metabolism, although other explanations of these events are certainly possible. It could be argued, though, that there is a physiologic incentive for such an interaction, since this mechanism of altering bone metabolism would permit a large marrow expansion under conditions of extreme hematopoietic stress. The local nature of this interaction is revealed by the fact that PTH levels were inversely related to bone resorption activity, implying that parathyroid gland activity was a reaction to, rather than a cause of, the high level of osteoclastic activity. Cross-reacting cellular interactions involving contrasuppression in the two relevant physiologic systems would certainly be consistent with this scenario.

If it is to be accepted that the BMSC and the BM contrasuppressor play a role in the regulation of hematopoiesis and bone metabolism, it remains to be explained why these cells can be detected in an in vitro immunological assay. Marluzzi (64) observed that the Thy-1$^-$ splenic suppressor cell found in ^{89}Sr treated mice was capable of suppressing in vivo PFC's only if the spleen cells were first passed through a 24 hr in vitro culture before transfer into the host mouse. This suggests that a selective differentiation occurs under in vitro conditions which leads to amplification of an immunologically suppressive cell. By extrapolation, it may be worthwhile to view the BMSC as not one cell but several closely related cells, perhaps arising from a common precursor cell which generates specific suppressors in accordance with the immediate physiologic stresses. Conversely, there may be a common BMSC whose activity within each physiologic sphere is regulated by one of an array of closely related Thy-1$^+$ contrasuppressors. While such arrange-

ments would help to account for the inter-system interactions, it is obvious that the specific cells being examined would be a function of the experimental design employed. Further dissection of this concept will require much more extensive phenotypic investigation of the BMSC and BM contrasuppressor.

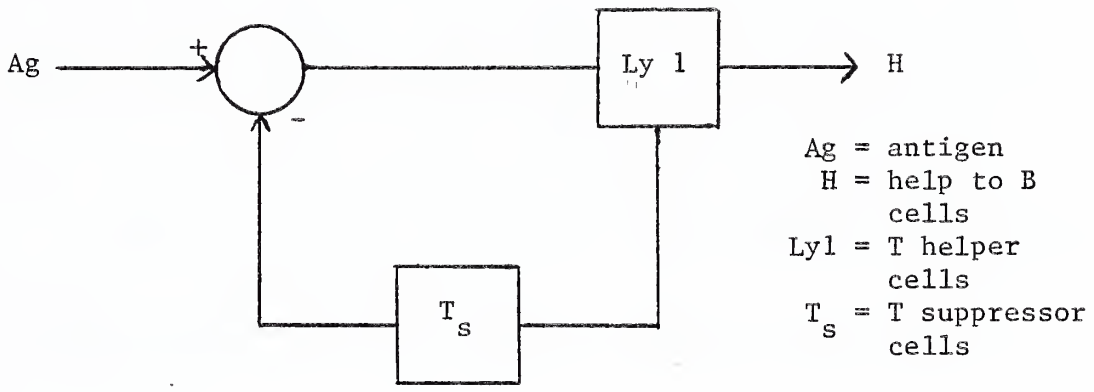
On a slightly more abstract level, this thesis represents the first report of contrasuppression in either the bone marrow or in a physiologic system free of exogenous influences. This cell, probably a lymphocyte, is Thy-1^+ but in the absence of proof of thymic dependence, it may be premature to call it a T cell (58,154,155). To the limited extent that it has been characterized, this cell is similar to the contrasuppressor found in an immunological assay involving neonatal spleen cells (15,16,41). In that experimental system there is a Thy-1^+, Ly-1^+, I-J^+ cell that acts to render T helper cells resistant to suppression by Ly-2^+ cells. In addition to a hypothetical global function for the contrasuppressor, such as in the hyperimmune state and in the transfer of delayed-type hypersensitivity, it has been suggested that contrasuppression may help regulate highly localized humoral immune responses (16). While this is probably not the case with the BM contrasuppressor, for the reasons discussed earlier in connection with BM antibody production, by expanding the domain of consideration to include hematopoiesis and bone metabolism it might be fair to view the BM contrasuppressor as a major determinant of the response to and control of micro-environmental changes.

As such, the greater significance of the BM contrasuppressor relates not to the specifies of its activities within each of its domains but, rather, to the light it sheds upon the interactions between

physiologic systems previously considered independent of one another. A multi-system physiologic response to various forms of stress is now a widely acknowledged paradigm, but without a greater understanding of this process on a cellular level rational attempts at medical intervention are impossible. This thesis sketches an integrated bone marrow physiology in which perturbations in local immune functions, hematopoiesis, or bone metabolism give rise to altered functional states in all three.

APPENDIX 1

In the basic feedback suppressor circuit, the cellular interactions can be diagramed as follows:



While such a representation ignores the details of the recruitment of lymphocytes from the pool of precursor lymphocytes, it is sufficient to display the major components of the feedback circuit. In simplest form, antigen (Ag) triggers T helper cells (Lyl) to aid in the production of antibody by delivering help (H) to B cells while a subpopulation of Lyl cells simultaneously triggers suppressor cells (T_s) which act to lessen the antigen responsiveness of T helper cells. Straightforward analysis of this feedback loop (156) yields the equation

$$H = Ag\left(\frac{Lyl}{1 + (Lyl)(T_s)}\right),$$

where H = help delivered
Ag = antigen
Lyl = T helper cell function
T_s = T suppressor cell function.

Thus, for given cellular response characteristics, it is possible to define the help delivered as a function of the antigen stimulus.

The concept of a contrasuppressor cell adds complexity to the system, but it too can be readily evaluated through similar block diagram analysis. Assuming that the contrasuppressor (CS) interacts within the Lyl - T_s domain, four different circuit possiblities can be envisioned. That is, the contrasuppressor can be stimulated by either

the Lyl or T_s cell and it could act upon either cell. These four basic circuits can be seen in Figure A, with the stimulant and acted-upon cells (with respect to the contrasuppressor cell) noted for each circuit.

On a theoretical basis, there is no cause to favor any one of the circuits described. However, experimental evidence (16) indicates that the contrasuppressor requires the presence of suppressor cells to be activated and that it acts to make T helper cells insensitive to suppression. These results are best accounted for by model IV in Figure A, although it is most definitely a very simplified representation of the cellular events involved.

By analyzing model IV, a mathematical representation can be derived as before:

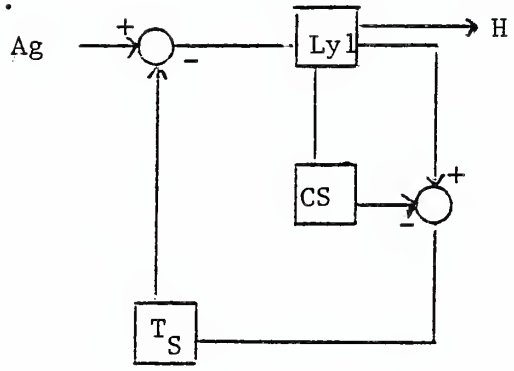
$$H = Ag\left(\frac{Lyl}{1 + (Lyl)(T_s - CS)}\right), \text{ where } CS = \text{contrasuppressor cell function.}$$

Ignoring the kinetics of the situation for a moment, it can be seen that as contrasuppression increases, between 0 and T_s, the denominator independently decreases, thereby increasing the magnitude of H (help) delivered by the circuit. The contrasuppressor, then, can be viewed as a fine-tuning gain control for the circuit as a whole. It can also be appreciated that, depending upon the particular kinetics of the cells involved, the contrasuppressor can also increase the responsiveness of the circuit and thereby allow a quick antibody response to the sudden introduction of an antigen that has been previously encountered. A more detailed exploration of this concept would be best accomplished through the use of an analogue computer.

Fig. A

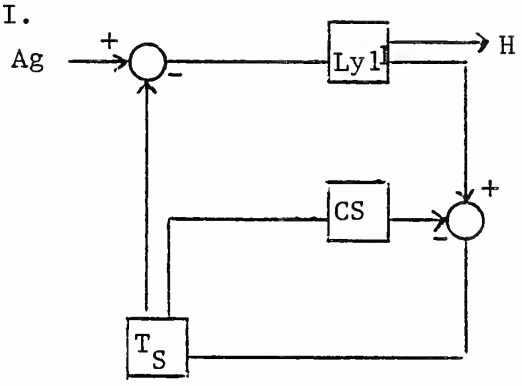
THEORETICAL CONTRASUPPRESSOR CIRCUITS

I.



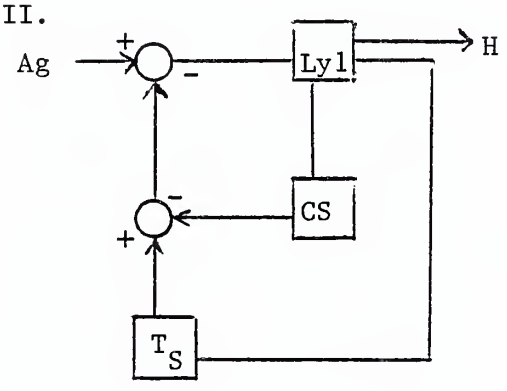
Source of stimulus - Ly1 cell
Site of action - T_S cell

II.



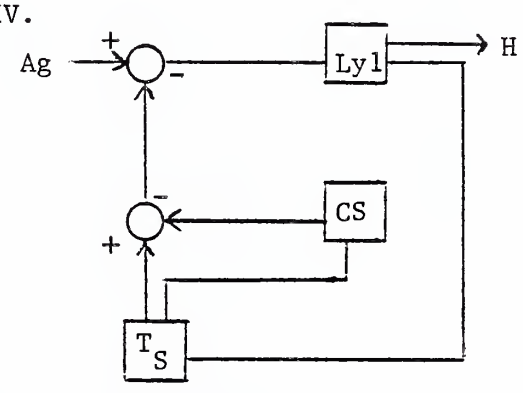
Source of stimulus - T_S cell
Site of action - T_S cell

III.



Source of stimulus - Ly1 cell
Site of action - Ly1 cell

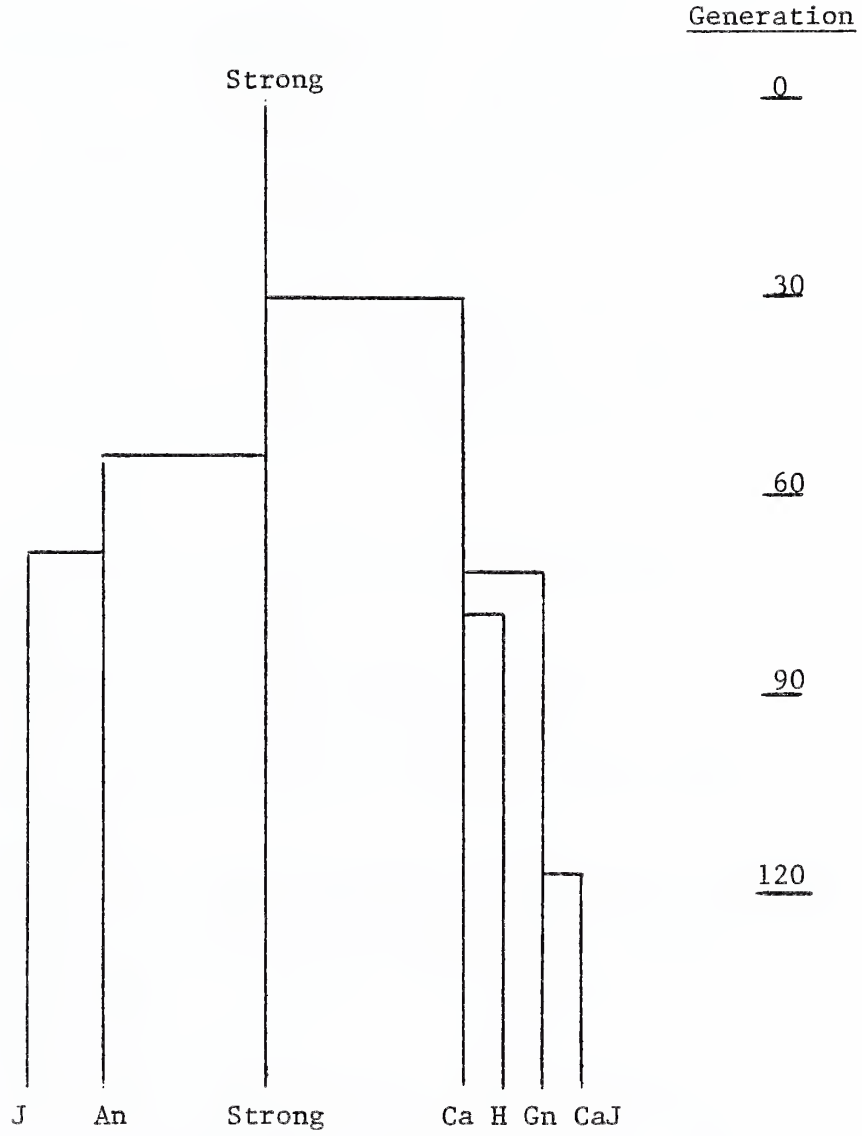
IV.



Source of stimulus - T_S cell
Site of action - T_S cell

APPENDIX 2:

GENEALOGY OF CBA SUBSTRAINS



Adapted from: Genealogies of Long-Separated Sublines in Six Major Inbred Mouse Strains; in Inbred and Genetically Defined Strains of Laboratory Animals. Part 1. Mouse and Rat. (P.L. Altman and D.D. Katz, eds.); Fed. Amer. Soc. Exp. Biol., Bethesda, Md,1978; p. 19.

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